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1. MacCorquodale, D. W.; Thayer, S. A., and Doisy, E. A.: J. Biol. Chem. 115: 435, 1936.

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ENDOCRINOLOGY

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PEROXIDASE ACTIVITY OF THE THYROID GLAND UNDER NORMAL AND EXPERIMENTAL CONDITIONS[1]

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IT WAS RECENTLY shown (De Robertis and Moura Gonçalvez' 1945) that in the thyroid gland in its normal state there is a wide difference between the oxidation-reduction potential of the follicular cells and of the colloid, and that this difference is altered when the gland is activated. Furthermore, it was found that thiourea produces a drop in the oxidation-reduction potential of the cells whereas sulfonamides have no effect whatsoever, in spite of the fact that both these substances exert a general inhibitory action upon thyroid function.

Dempsey (1944), using histochemical methods, recently discovered in the normal thyroid cells a peroxidase activity which is inhibited by thiouracil. This observation is particularly important because the peroxidases catalyze the liberation of iodine from iodides and their presence in the cells may be related to biological iodinations (Keston, 1944), and to the formation of thyroxine (Westerfeld and Lowe, 1942). Glock (1944), however, as the result of his biochemical studies denies that there is a true peroxidase in the thyroid gland, and favors the idea that peroxidase activity is due to the non-specific action of hemoglobin. These contradictory results have led us to investigate the enzyme systems which could be involved primarily in the activation or inhibition of the thyroid gland by thiourea and by sulfonamides.

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METHOD

Experiments were carried out mostly on thyroid glands of young albino rats. A few canine and human glands were also studied. As activators the influence of cold and the injection of Antuitrin T (thyrotropic hormone of the pituitary gland) were used, and as inhibitors, thiourea and sulfonamides both *in vitro* and *in vivo*. Peroxidase activity was determined by the oxidation of Nadi reagent, benzidine, guaiacol, pyrogallol and o-phenylenediamine in the presence of hydrogen peroxide, and also by the liberation of free iodine from iodide. The methods varied in each series of experiments and will be described with the results. Control experiments were carried out with hemoglobin, with potato extracts, and with extracts of various tissues known to possess no peroxidase activity (Bancroft and Elliot, 1934).

RESULTS

Peroxidase activity of the follicular colloid studied by the microdissection technique. The technique used was, with slight modifications, similar to that previously described (De Robertis, 1941). Rats were

TABLE I. INDOPHENOLOXIDASE AND PEROXIDASE ACTIVITY IN THE COLLOID EXTRACTED BY MICRODISSECTION

	Normal rats	Activated by cold	Activated by TSH	Action of thiourea on activated glands	Action of KCN on activated glands
Indophenol oxidase	−	−	−		
Peroxidase	−	+	+	−	−
E_0' at pH 7 (approx. in volts)	−0.200	+0.050	+0.050	−0.200	−0.200

−, negative, +, positive. E_0', oxidation-reduction potential. TSH, thyrotropic hormone.

anaesthetized with Nembutal. The isthmus of the thyroid gland was exposed and observed under a magnification of 100×. Micropipettes with a bore of 5 to 10 microns at the tip were adapted to a microinjector filled with mercury and guided by a micromanipulator. At the beginning of the experiment a small quantity of the reagent was sucked in (approximately 2×10^{-6} ml.). Control experiments were made in which the tip of the pipette was filled with mineral oil, in order to avoid contact of the reagent with the mercury, but no difference in the results was observed.

Nadi reagent was used to demonstrate indophenol oxidase, and Nadi reagent with hydrogen peroxide was used to investigate peroxidase activity. Alpha-naphthol was dissolved in alcohol and p-phenylenediamine was dissolved in a buffered phosphate of pH 7.0. The follicle selected was punctured and the colloid aspirated. As the reagent and the colloid did not mix, they remained separated by an interphase.

In Table I the results are set forth and compared with the oxidation-reduction potentials. Indophenol oxidase was negative both in normal and in activated glands. Peroxidase was negative in normal

glands but in tests on rat thyroids activated by cold or by the injection of 5 units of thyrotropin, a violet color developed at the interphase in about one minute, indicating the presence of peroxidase. Thiourea inhibited the reaction of activated glands in a concentration of 0.005M or by the injection of 8–10 milligrams of thiourea. 0.01M of potassium cyanide also inhibited the reaction.

Peroxidase activity of thyroid cells and colloid determined by the supravital technique. Rats were killed by a blow on the head and were immediately exsanguinated by opening the chest. The thyroid glands were dissected out with bone forceps and glass needles and were then divided (in Ringer's solution at pH 7) into small portions, using glass needles, which made it possible to see the follicles clearly under the dissecting microscope. Nadi reagent in a concentration of 0.01M was dissolved in Ringer's solution and a small amount of hydrogen peroxide added. The results are summarized in table II.

TABLE II. PEROXIDASE ACTIVITY OF CELLS AND COLLOID, DETERMINED WITH THE SUPRAVITAL TECHNIQUE

	Normal		Activated		Action of KCN		Action of thiourea		Action of sulfanilamide	
	cells	colloid	cells	colloid	cells	colloid	cells	colloid	cells	colloid
Peroxidase	+	−	+	+	−	−	−	−	+	−
E_{h} at pH 7 (in volts approx.)	+0.050	−0.200	+0.050	+0.050	−0.200	−0.200	−0.200	−0.200	+0.050	−0.200

Normal glands gave a very intense reaction, with a reddish color visible under the microscope after 30 seconds, and easily seen with the unaided eye after 60 seconds; in 5 to 10 minutes the color was very intense, becoming bluish. The test for indophenol oxidase was at first negative, but after 5 minutes a pale red color developed. In contrast with this, in any organ rich in cytochrome oxidase, such for instance as the heart, the reaction appeared in one minute and was very intense in 5 minutes. In the thyroid gland peroxidase activity was very strong, as indicated by the oxidation of pyrogallol, benzidine, o-phenylenediamine and guaiacol. These reactions were negative in the absence of hydrogen peroxide. 0.01M of potassium cyanide completely inhibited the peroxidase activity. Glands activated with thyrotropin gave an intense reaction both in the cells and in the colloid.

Thiourea had an evident inhibitory action upon the peroxidase activity, both *in vitro* and *in vivo*. With a concentration of 0.01M the reaction was negative for 5 to 7 minutes, but after this time a pale red color appeared. This effect may have been due to cytochrome oxidase, because 0.01M thiourea had no inhibitory effect whatsoever on the indophenol reaction of cardiac tissues.

Rats were injected with 35 mg. of sulfanilamide and two hours later the glands were removed and put in a 0.005M solution of this drug. The peroxidase reaction was not affected by this treatment. It is

interesting to note that thyroid glands of rats injected with the sulfanilamide showed a reddish color.

Peroxidase activity studied in frozen sections. Thyroid glands were frozen in liquid air or in solid CO_2 and cut 20 to 40 microns thick with the freezing microtome. Sections were kept in 0.9% NaCl cooled with ice, and put for 5 minutes in a 0.1% solution of ammonium molybdate dissolved in 0.9% NaCl; this treatment, as shown by Mancini and Celani (1940), intensifies the reaction. The sections were in turn put in a saturated solution of benzidine in 0.9% NaCl to which a drop of hydrogen peroxide (20 vol.) per 2 ml. had been added. The sections were moved about in the solution with a glass rod until the reaction appeared (30 to 40 seconds). After 2 minutes the sections were washed in 0.9% NaCl. A blue color was observed in the tissue whereas the colloid remained clear. This color was due to the presence of small refringent blue granules distributed in the cytoplasm with a tendency to condense about the nucleus. Around the follicles, intensely stained mast cells appeared, and sometimes a few intensely colored erythrocytes were seen inside the capillaries. Table III shows the results obtained in normal sections and in the presence of thiourea and sulfanilamide.

TABLE III. ACTION OF THIOUREA AND SULFANILAMIDE IN FROZEN SECTIONS

	M 0.01	M 0.005	M 0.0025	M 0.002	M 0.001	M 0.0001
Control	++					
Thiourea	−	−	−	−	+	++
Sulfanilamide	+++	+++				

Thiourea completely inhibited the reaction, in a concentration of 0.002M, while with 0.001M the reaction was weaker than in the controls. On the other hand, the erythrocytes gave an intense reaction even in a 0.01M solution. Sulfanilamide not only did not inhibit the reaction but actually intensified it.

The action of thyroid extract upon the liberation of iodine from iodide. Experiments were carried out on the thyroid glands and other organs of dogs which were exsanguinated and perfused with 0.9% NaCl until all the blood was washed out of the body. The glands were ground in a mortar and extracted for 24 hours with 60% glycerol in the proportion of 0.05 ml. per 100 mg. of tissue. The extract was filtered through gauze and dialyzed through cellophane of 4°C. during 24–48 hours. The final volume was diluted to a concentration equivalent to one-fourth of the initial concentration. Control extracts of brain and muscle were prepared by a similar technique, and a very dilute solution of freshly hemolized blood and a solution of crystalline hemoglobin were also used as controls. In addition an aqueous extract of potato containing peroxidase was tested.

In each experiment 0.5 ml. of the extract and 0.25 ml. of 0.01M

KI were put into small tubes and the volume brought up to 1 ml. with water or with the various reagents used. One drop of H_2O_2 (20 vol.) was added and experiments were carried out at room temperature.

Because hydrogen peroxide can directly liberate free iodine from iodide, several experiments were made to study the behavior of this reaction in the presence of thiourea or sulfonamides. The reaction increased progressively with time and a yellow color was developed which was due to iodine, as could be shown with starch as indicator. Cyanides and thiourea in concentrations stronger than 0.001M slowed the reaction. In concentrations of 0.01M and 0.03M respectively they stopped it. Sulfathiazole had a completely different action. In the test tube a yellow color appeared which increased continuously and then formed a precipitate, but the reaction itself was completely negative in concentrations ranging from 0.03M to 0.001M.

In the presence of thyroid extracts, iodine was also liberated but the reaction had different characteristics. It started rapidly and intensified but reached a maximum in two or three minutes and then apparently stopped. The color developed never reached the same intensity as in the direct reaction. Complete inhibition of the liberation of iodine was obtained with thiourea until a concentration as low as 0.001M was reached (Table IV). If the extract was heated to 100°C. the reaction followed the direct type and was not inhibited by 0.01M thiourea (Table IV).

TABLE IV. ACTION OF THIOUREA ON THE LIBERATION OF IODINE FROM IODIDE

	Control	M 0.03	M 0.01	M 0.005	M 0.0025	M 0.002	M 0.001	M 0.0001
Thyroid extract	+		−	−	−	−	−	+
id. heated at 120°C.	+		+	+	+	+	+	+
Potato extract	+		−	−	−	−	±	+
id. heated at 120°C.	+		+	+	+	+	+	+
Hemoglobin	+	−	+	+	+	+	+	+
id. heated	+		+	+	+	+	+	+
Brain extract	+		+	+	+	+	+	+
Muscle extract	+		−	+	+	+	+	+

Vegetable peroxidase obtained from a potato extract gave with thiourea results similar to that of the thyroid extract. On the contrary, hemoglobin gave a reaction of the direct type which was only inhibited by thiourea in a concentration of 0.03M.

Since perfused brain and muscle have no peroxidase activity (Bancroft and Eliott, 1934) control experiments were done with extracts of these tissues. As with hemoglobin the reaction was of the direct type and was not inhibited by weak concentrations of thiourea (Table IV).

Experiments made with potassium cyanide gave results almost

similar to those with thiourea. Sulfathiazole apparently stopped the liberation of iodine up to a concentration of 0.001M, but as in the case of the direct reaction a yellow compound was formed. As seen in table V, potato extract and hemoglobin had a similar effect.

TABLE V. ACTION OF SULFATHIAZOLE ON THE LIBERATION OF IODINE FROM IODIDE

	Control	M 0.01	M 0.005	M 0.0025	M 0.002	M 0.001	M 0.0001
Thyroid extract	+	−	−	−	−	−	+
Hemoglobin	+	−	−	−	−	−	+
Potato extract	+	−	−	−	−	−	

DISCUSSION

The discovery that substances such as thiourea and sulfonamides inhibit the thyroid secretion (Mackenzie, Mackenzie and McCollum, 1941; Kennedy, 1942; Richter and Clisby, 1942; and Astwood, Sullivan, Bissel and Tyslowitz, 1943) led to the investigation of the enzyme systems which could be involved in the oxidation-reduction potentials of the thyroid gland and of the manner in which the mechanism of synthesis of the thyroid hormones may be altered by these drugs.

Experiments *in vivo* and *in vitro* with radioactive iodine have clearly shown that sulfonamides and thioureas inhibit the conversion of inorganic iodine into organic compounds, so that the formation of diiodotyrosine and thyroxine is almost suppressed (Franklin and Chaikoff, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Coucero, 1944; Franklin, Chaikoff and Lerner, 1944; and De Robertis, Chagas, and Coucero, 1945). There are many reasons which lead us to assume that the biological synthesis of thyroxine occurs in at least two phases: first, liberation of iodine from iodide and iodination of tyrosine into diiodotyrosine, and second, the union of two molecules of diiodotyrosine to form thyroxine (Harrington, 1944). We must suppose that the treatment with sulfonamides and thioureas suppresses both these stages or at least the first one. Johnson and Tewkesbury (1942), found that the synthesis of thyroxine *in vitro* is inhibited by sodium sulfite. This result led to the conclusion that oxidative processes are involved in the synthesis, the oxidative agent being iodine or hypoiodous acids. Furthermore Li (1942) found that the rate of iodination of thyroxine to 3–5 diiodotyrosine is a function of the concentration of hypoiodous acid and iodine. Using I^{131} as indicator, Schachner, Franklin, and Chaikoff (1943) found that the formation of diiodotyrosine and thyroxine *in vitro* is inhibited by cyanide, azide, sulfide, and carbon monozide. They reached the conclusion that in these processes the cytochrome-cytochrome oxidase system is involved, but they pointed out that other enzyme systems such as peroxidase could not be ruled out.

Westerfeld and Lowe (1942), studying the oxidation of p-cresol by peroxidase, suggested that the quinol-ether linkage of thyroxine may be formed under the action of a peroxidase. These ideas, together with the previously known facts that thiourea has an inhibitory effect on peroxidase (Sumner and Somers, 1943) and that sulfonamides, although they do not produce inhibition (Mann and Keilin, 1940) have a competitive action upon vegetable peroxidase reactions (Wood, 1940 and Lippmann, 1941), gave importance to the experiments of Dempsey (1944), who discovered a peroxidase reaction in thyroid cells which is inhibited by thiouracil. Glock (1944), however, questioned these results and by biochemical methods reached the conclusion that peroxidase activity of the thyroid gland is due to the presence of hemoglobin.

The results reported here are related to our previous work (De Robertis and Moura Gonçalvez, 1945) on the oxidation-reduction potential of the thyroid follicle. In the colloid extracted by microdissection from follicles of glands activated by cold or by the thyrotropic factor of the pituitary gland there is a peroxidase reaction; the latter is negative in normal glands and becomes negative in activated glands treated with thiourea. In this connection it is interesting to mention that in sections of thyroid glands stimulated by thyrotropin, Okkels (1934) has described the penetration of oxidative granules into the colloid.

In experiments with the supravital technique in frozen sections, using benzidine and other phenol compounds such as pyrogallol, p-phenylenediamine, Nadi reagent, and guaiacol, we have observed (confirming Dempsey's results) a peroxidase reaction in the cells. In the activated glands and in glands under the action of potassium cyanide, thiourea and sulfonamides, the results were in accordance with the oxidation-reduction potentials, so that when the potential was high (+0.050 volts) the reaction was positive, when it was low (−0.200 volts) the reaction was negative.

In order to check the specificity of the peroxidase reaction, experiments were made on thyroid glands of exsanguinated rats and of dogs which were exsanguinated and perfused. In these glands, in spite of the removal of the blood the reaction was still present in the cells. Furthermore, in order to differentiate pure peroxidase from pseudoperoxidase (such as hemoglobin) sections were treated at 120°C. for 20 minutes. The reaction disappeared from the cells but persisted in the erythrocytes. We have also confirmed Dempsey's observations that a much greater concentration of the drug (thiourea) is needed to inhibit the reaction of the red blood cells.

Since Glock questioned the specificity of the peroxidase activity because of the strong concentration of thiourea or thiouracil needed to inhibit it in the thyroid gland of the horse, a few experiments were carried out with thyroids of exsanguinated dogs. With a simplified

purpurogallin method we found a much stronger inhibition than was reported by Glock (Table VI).

With the benzidine reaction, applied to sections, we found that thiourea completely inhibits the reaction up to a concentration of 0.002M and that the reaction is still much weaker than in the normal with 0.001M. On the contrary sulfanilamides not only do not inhibit the reaction but even intensify it. These results confirm our previous observation that sulfa-compounds do not change the oxidation-reduction potential of the follicle as does thiourea. They are in complete agreement with the results previously reported on vegetable peroxidase by the action of thiourea (Mann and Keilin, 1940) and sulfa-

TABLE VI

Glock 1944		De Robertis-Grasso	
Concentration of drug	Inhibition	Concentration of drug	Inhibition
0.08 M	96%	0.005 M	69%
0.016 M	55%	0.0005 M	38%
0.0016 M	5%		

compounds (Mann and Keilin, 1940; Wood, 1940; and Lippmann, 1941). The apparent discrepancy in the action of these two kinds of thyroid inhibitors is very interesting, especially because both affect the biosynthesis of the thyroid hormone in a rather similar way (Franklin and Chaikoff, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Coucero, 1944; Franklin, Chaikoff and Lerner, 1944; De Robertis, Chagas, and Coucero, 1945).

The difference in the mechanism of action of sulfonamides and thiourea is particularly manifest in the experiments on the liberation of iodine. In the presence of potato extracts or thyroid extracts (and also directly when the hydrogen peroxide acts on KI), liberating iodine, sulfathiazole apparently forms a new compound, and free iodine cannot be found. This result may be related to the appearance of a reddish tinge in the thyroid gland after the injection of sulfanilamide. The action of sulfonamides seems to be due to competitive action by which the liberated iodine attaches itself to the sulfa-compounds instead of tyrosine. This interpretation is in agreement with the previously reported results on vegetable peroxidase (Wood, 1940; Lippmann, 1941).

Our results lead us to the conclusion that thiourea acts by inhibiting the peroxidase system which takes part in the liberation of iodine from iodides, thus preventing the formation of free iodine necessary for the iodination of tyrosine to 3–5 diiodotyrosine.

In this connection it is interesting to note that thiourea has no action on the proteolytic system of the thyroid which is related to the reabsorption of the colloid and probably to the secretion process (De

Robertis and Nowinski, 1946). Experiments made in this laboratory by Grasso (unpublished) show that thiourea does not interfere with the secretion of colloid droplets by the cells or with the reabsorption of the follicular colloid.

SUMMARY

Peroxidase activity is histochemically demonstrable in the cells of the thyroid gland, by the oxidation of benzidine, Nadi reagent, o-phenylenediamine, guaiacol, and pyrogallol and by the liberation of iodine from iodide. This activity disappears when the tissue is heated to 120°C. It is specific and not due to hemoglobin.

In thyroid glands activated by cold or stimulated with thyrotropic hormone, the peroxidase reaction becomes positive in the colloid also. This change is related to the increase of the oxidation-reduction potential, as previously reported (De Robertis and Moura Gonçalvez, 1945).

Thiourea has an inhibitory effect upon the peroxidase activity (in concentrations of 0.001M) while sulfa-compounds have no effect. This difference is also related to changes in the oxidation-reduction potential of the cells when similarly treated.

Thiourea has an inhibitory effect upon the liberation of iodine from iodide (also in concentrations of 0.001M) while sulfathiazole seems to influence the utilization of iodine, liberated from iodide, in the formation of new compounds.

The inhibitory effect of thiourea on the function of the thyroid gland is probably due to direct inhibition of the peroxidase system responsible for the liberation of iodine from iodide, thus preventing the iodination of tyrosine to 3–5 diiodotyrosine.

The inhibitory effect of sulfonamides upon the function of the thyroid gland is not due to the inhibition of the peroxidase system but to the competitive action of these drugs by which free iodine attaches itself to them rather than to tyrosine.

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EFFECT OF THIOUREA ON BODY FAT AND LIVER GLYCOGEN OF RATS

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NUMEROUS PAPERS have been published in the past few years showing that thiourea suppresses the formation of thyroxin by the thyroid gland, although the exact mechanism has not been fully explained. Since thyroxin is such an important metabolic stimulant, the experiments here reported were started to determine whether this suppression of thyroxin formation affected the neutral fat and cholesterol content of the animal's bodies, skins, and livers. Studies are also reported on the possible inhibiting action of the feeding of dried thyroid gland on the changes from the thiourea administration. In one experiment liver glycogen was also determined since the histological picture of the liver indicated a high glycogen content of the livers of the thiourea fed animals.

Young Albino rats of the Wister strain were used in all the experiments. In experiment 1 the animals were fed Rockland rat diet without additional vitamin supplements. Diets of known composition were used in experiments 2–10. In experiments 2–4 and 6–8 the basic diet consisted of:

Sucrose 71.3 gm.
Casein (Vitamin free) 20 gm.
Salt Mixture without KI (Wesson 1932) 2.7 gm.
Corn Oil 5 gm.
Cellu Flour (B) 1 gm.
Potassium Iodide 1.8 mg.

Vitamin supplements were added to each 100 grams of ration as follows: Thiamine hydrochloride 4 mg., riboflavin 8 mg., pyridoxine hydrochloride 6 mg., calcium pantothenate 15 mg., niacin 14 mg., choline chloride 100 mg., percomorph oil, 50 per cent, 15 drops. In addition, each rat received 2.5 mg. of alpha tocopherol twice a week. The dried thyroid gland was always fed at a level of .005 per cent. Preliminary experiments showed that this amount of thyroid was sufficient to allow moderate growth of thiourea fed aimals on an unrestricted diet.

The low protein diet used in the other experiments was similar to

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the above basic diet except that the protein content was reduced to 10 per cent and the carbohydrate correspondingly increased. The high protein diet was similar except that it contained 24 per cent protein and only 67.3 per cent sucrose.

In the individual experiments from 1–4 the same amount of food was fed per 100 grams of body weight, the amount being regulated to that which those eating the least were expected to consume. The general appetite of the controls was always definitely better than that of the thiourea fed animals. They always ate all of their food while some of those receiving thiourea sometimes would leave a part of theirs. In experiments 5–10 food intake was controlled so that all the animals in each experiment ate the same amount of food. Litter mates of the same sex and weight were taken for both control and experimental animals. The food fed each day was the exact amount the animal, eating the least, consumed the day before.

The animals throughout all experiments were kept in an air conditioned room with a temperature of approximately 77°C. At the end of the experimental period the animals were rapidly exsanguinated by decapitation, their livers were removed and individually weighed. In some cases sections were taken for histological studies after staining with either eosin and hemotoxylin for general examination or with Best-Carmine stain for glycogen in experiments 6, 9 and 10. The remaining portion of the liver was ground in a mortar and after thorough mixing, samples were taken for lipid analyses using the method of Outhouse and Forbes (1940). Liver glycogen was determined in experiment 10 by the procedure of Goode *et al.* (1933) The bodies in each group, after removing the skin and intestinal tract, were ground in a meat chopper, transferred to a weighed beaker, and dried in an oven at 100°C. In experiments 6–10 the skins from each group were similarly treated except they were not ground prior to drying. After thorough drying, the bodies were ground in a large mortar, thoroughly mixed, and samples taken for analyses. The skins were put through the meat chopper after drying, following this they were thoroughly pounded in a mortar. Thorough mixing of the skins was impossible, therefore the values obtained are considered as approximate only.

DISCUSSION

It will be seen from the results which are recorded in Table 1 that the administration of thiourea did not affect the distribution of either neutral fat or cholesterol in the animal's liver or body. The neutral fat and cholesterol content of the skins was determined only in experiments 6–10. In these, without a single exception, the neutral fat content of the skins of the animals receiving thiourea was slightly higher than that of the controls. This was the case whether dried thyroid was or was not given. The skin values, however, are not inincluded in the table since the differences were not marked and the

TABLE 1

Exp. number	Days on diet	Number of rats	Average initial wt.	Average daily gain	Liver			Body		Diet used
					Per cent body wt.	Neutral fat	Cholesterol	Neutral fat	Cholesterol	
			gm.	gm.		%	%	%	%	
1	54	3	66	1.4	4.7	2.7	0.23	12.6	0.42	Rockland rat diet
		4	62	0.4	5.2	2.3	0.37	8.0	0.49	Same+1% thiourea
		4	62	0.5	5.2	2.4	0.27	7.5	0.49	Same+0.5% thiourea
		4	60	0.8	4.7	3.0	0.26	10.5	0.49	Same+0.1% thiourea
2	47	4	72	2.3	4.6	2.3	0.23	29.0	0.44	Basic diet
		4	77	0.8	5.7	1.2	0.21	17.6	0.40	Same+0.5% thiourea
		4	77	1.1	4.5	1.4	0.25	18.2	0.32	Same+0.5% thiourea+10% yeast
		4	71	1.3	5.7	2.1	0.27	18.6	0.42	Same+0.1% thiourea
		4	66	1.2	5.1	1.5	0.25	18.6	0.36	Same+0.1% thiourea+10% yeast
3	43	4	79	2.2	4.2	2.9	0.26	22.1	0.30	Basic diet
		4	87	0.9	5.8	4.6	0.30	20.7	0.32	Basic diet+0.25% thiourea
		2	80	0.2	5.4	1.5	0.21	19.7	0.44	As above+10% yeast
4	50	5	56	2.7	4.4	3.1	0.17	27.0		Basic diet
		5	51	0.3	5.7	2.5	0.16	16.5		Basic diet+0.5% thiourea
		5	53	1.0	6.0	5.9	0.25	15.0		As above+0.005% thyroid
		3	52	1.8	6.2	3.1	0.22	16.4		Basic diet+0.5% thiourea+1% yeast
5	14	6	143	0.5	4.5	4.6	0.26			Low protein diet
	14	3	152	−1.0	5.7	2.1	0.34			Same+0.5% thiourea
	30	7	157	−0.1	4.8	3.9	0.18			Basic diet+0.5% thiourea
	30	5	156	−0.3	5.7	7.9	0.17			Same+1% thiourea
	30	4	156	−0.6	4.5	13.3	0.25			Same+1.5% thiourea
6	60	4	72	0.6	4.3	2.2	0.65	19.6	0.57	Baisc diet
		5	72	0.6	6.7	1.3	0.59	14.0	0.53	Same+0.5% thiourea
		4	72	0.6	6.8	1.9	0.65	17.3	0.61	As above+0.005% thyroid.
7	59	3	69	0.9	4.4	4.8	0.35	15.2	0.59	Basic diet
		3	69	0.9	7.3	2.0	0.37	17.8	0.46	Same+0.5% thiourea+0.005% thyroid
8	36	2	80	0.66	4.2	4.5	0.40	17.0	0.52	Basic diet
		2	80	0.62	5.9	1.7	0.64	17.0	0.58	Same+0.5% thiourea+0.005% thyroid
9	30	2	174	−0.47	4.2	1.6	0.25	13.0	0.46	Low protein diet
		2	176	−0.27	4.5	0.8	0.21	10.7	0.43	Same+0.5% thiourea
		2	177	−0.33	3.9	1.6	0.27	10.7	0.38	High protein diet
		2	174	0.00	4.9	0.7	0.18	12.3	0.43	Same+0.5% thiourea
10	30	5	73	0.01	4.9	3.5	0.34	13.3	0.81	Low protein diet
		5	72	0.01	5.7	1.4	0.30	16.1	0.65	Same+0.5% thiourea
		5	72	0.71	4.3	0.6	0.33	9.9	0.70	High protein diet
		5	71	0.33	4.7	0.9	0.30	13.4	0.72	Same+0.5% thiourea
					Liver Glycogen					
						1.72%				Low protein diet
						6.73%				Same+0.5% thiourea
						0.78%				High protein diet
						1.42%				Same+0.5% thiourea

values obtained are considered approximate only due to the uncertainty of sampling. Additional work using a much larger series of animals is necessary in order to determine whether the skin differences noted are significant or not.

The average liver weight, in the percentage of body weight, was higher, without exception, in those animals receiving moderate amounts of thiourea than in the corresponding controls. This is in contradiction to the conclusions arrived at by Leathem (1945). However, examination of his published data does show that the average liver weight of his thiourea fed animals also was slightly greater than that of his control animals. It is probable that if his experiments

were continued for a longer period of time the differences would have approached those obtained by us. The administration of thyroid apparently does not influence the effect of the thiourea on liver weight, as shown in experiment 5. Yeast, however, in the absence of thyroid seems to have exerted some inhibiting action.

Examination of the eosin and hemotoxylin stained sections showed no evidence of liver damage in any of the animals, in complete agreement with the results of Leathem. The sections, however, always suggested a higher liver glycogen content of the livers of the thiourea fed animals than those of the corresponding controls. This was con-

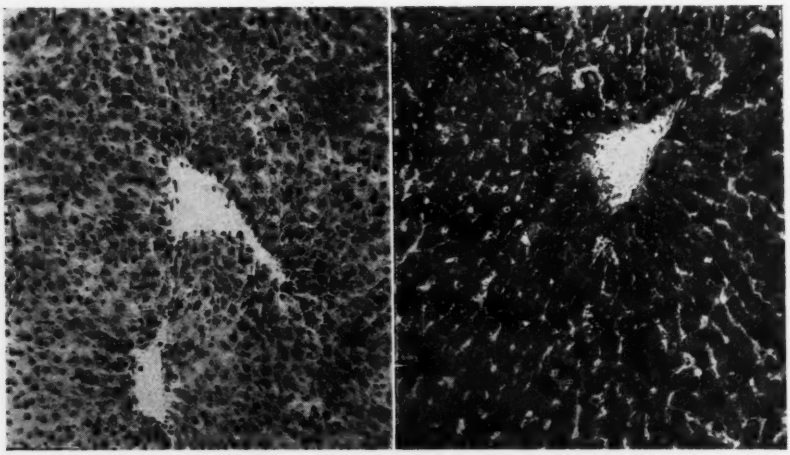

FIG. 1

Control animal Thiourea fed animal
Sections of liver stained for glycogen with Best-Carmine.

firmed by Best-Carmine staining of the liver for glycogen in later experiments, and by glycogen analyses in experiment 10. Figure 1 shows typical glycogen stained sections from experiment 9. It will be seen that the controls show little glycogen while the thiourea fed animals show marked deposition. This effect of thiourea on liver glycogen concentration may be due to diminished glycogenolysis through inhibition of epinephrine action, as Friedenwall and Buschke (1944) showed that thiourea inhibited the effect of epinephrine on the rabbit's nonpregnant uterus and small intestines.

The increased relative size of the liver in the thiourea fed animals can be to some extent explained on their increased glycogen content. Water content does not seem to play a role as, in experiment 9, the water content of the livers was determined and no differences between the groups could be noted. Variations in the fat content also is not a factor. The absence of any inhibiting effect of thyroid feeding on

the increase in liver weight resulting from thiourea administration indicates that this thiourea effect cannot be due to its well known inhibiting action on thyroxin formation.

SUMMARY

Thiourea administration does not affect the distribution of neutral fat and cholesterol in the livers or the skinned bodies of rats. Its administration leads to a relative increase in liver weight and to a marked increase in liver glycogen content.

Thyroid administration did not influence the effect as long as food intake was kept the same for both controls and experimental animal .

It is suggested that the high liver glycogen of the thiourea fed animals may be due to diminished glycogenolysis through inhibition of epinephrine action.

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EFFECT OF THIOURACIL AND OTHER COMPOUNDS ON THE SUCCINOXIDASE SYSTEM OF RAT THYROID TISSUE

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IT WAS REPORTED recently that cytochrome oxidase of thyroid tissue is inhibited by thiouracil, certain sulfonamides and other compounds such as glutathione and ascorbic acid (Paschkis, Cantarow, and Tillson, 1945). The latter compound was reported to inhibit completely. The basis for this inhibition was that less color was developed when thyroid tissue reacted with paraphenylenediamine in the presence than in the absence of these compounds. The free radicals derived by the univalent oxidation of paradiamines such as paraphenylenediamine are known as Wurster's salts, and the color and stability of certain of these radicals have been compared by Michaelis, Schubert and Granick (1939). In this connection Potter (1942) has also shown that certain sulfhydryl-containing enzymes such as urease and succinic dehydrogenase are inhibited by split products of paradimethylaminoazobenzene (diamines) when they are oxidized, and the inhibition was correlated with both the autoxidizability of the diamines and their free radical stability.

In the light of the fact that ascorbic acid has been used as a substrate in the presence of excess cytochrome c for the determination of the activity of cytochrome oxidase (Schneider and Potter, 1943), it seemed that this acid should not inhibit the enzyme in the presence of paraphenylenediamine as substrate. Consequently we have studied the effect of sulfaguanidine, sulfathiazole, thiourea, thiouracil, cysteine and glutathione on the cytochrome oxidase activity of thyroid tissue of male rats when ascorbic acid was used as substrate in the presence of added cytochrome c. The composition of the fortified substrate is given in footnote 1 of table 1. A homogenate of the tissue was prepared according to the method of Potter and Elvehjem (1936).

The effect of the same compounds on the activity of succinic dehydrogenase of rat thyroid tissue was determined by using sodium succinate as substrate in the presence of added cytochrome c (Potter and Schneider, 1942). The composition of the substrate for this determination is given in footnote 2 of table 1.

The final molarity of the compounds used in the Warburg flasks

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in testing for inhibition is given in table 1. Oxygen uptake was obtained by use of a conventional Warburg apparatus. The experiments were run at 38° C. and readings were made at 10 minute intervals. These readings were used in the calculation of the Q_{O_2} (cmm. of oxygen taken up per mg. of dry tissue per hour).

Results and Discussion. The results given in table 1 show that reduced glutathione in a concentration of 0.001 M inhibited cytochrome oxidase to the extent of approximately 50 per cent. Thiourea caused slight inhibition which confirms the results reported by Graubard (1940–41) for uterine tissue when paraphenylenediamine was used as

TABLE 1. RESULTS SHOWING EFFECT OF SULFONAMIDES AND OTHER COMPOUNDS ON THE SUCCINOXIDASE SYSTEM OF THYROID TISSUE

	Q_{O_2} for cytochrome oxidase[1]								Kind of rats	
									Age Mo.	Bd. wt. gm.
Compound tested for inhibition	Control (no inhibitor)	Sulfaguanadine	Sulfathiazole	Glutathione	Cysteine	Thiourea		Thiouracil	—	—
Final molarity of inhibitor	—	0.002	0.001	0.001	0.002	0.002	0.004	0.002	—	—
Experiment 1	161.4	164.4	—	82.2	163.6	155.1	—	166.1	5–6	400
Experiment 2	200.4	202.1	200.7	110.3	190.4	—	189.0	213.3	4–6	390
	Q_{O_2} for succinic dehydrogenase[2]									
Experiment 3	8.8	9.4	8.3	9.6	8.9	9.7	—	7.4	4–6	380
Experiment 4	15.9	16.2	15.9	16.4	16.4	17.1	—	15.4	1–2	113

[1] The substrate solution consisted of 0.3 ml. of 0.114 M ascorbic acid, 0.3 ml. of 9×10^{-4} M cytochrome c, 0.3 ml. of 0.004 M $CaCl_2$, 0.3 ml. of 0.004 M $AlCl_3$, 1 ml. 0.1 M phosphate buffer of pH 7.4, 0.2 ml. of 3 per cent homogenate of thyroid tissue, 0.3 ml. of inhibitor solution to be tested and sufficient water to make a total volume of 3 ml. (Schneider and Potter, 1943).

[2] The substrate solution consisted of 0.3 ml. of 0.5 M sodium succinate, 0.2 ml. of 3×10^{-4} M cytochrome c, 0.3 ml. of 5 per cent homogenate of thyroid tissue, and the same amounts of the other solutions as was used for cytochrome oxidase. One tenth ml. of 20 per cent KOH was used in the center cups in all cases (Potter and Schneider, 1942). The compounds tested for inhibition and the tissue homogenate (enzyme) were added to the fortified substrate at approximately the same time and immediately before the beginning of the experiment.

substrate. The almost complete inhibition with glutathione and complete inhibition with ascorbic acid which Paschkis et al reported can be explained on the basis that these two compounds decolorize the colored compound formed from paraphenylenediamine by the cytochrome-cytochrome oxidase system of the tissue. That there is a direct action of these compounds on the colored compound was shown by oxidizing paraphenylenediamine to the colored form with bromine, and later adding ascorbic acid or glutathione which in the case of the former decolorized completely, and in the case of the latter there was about 50 per cent reduction in color (table 2). This shows that the "inhibition" reported by Paschkis et al is explainable on the basis of interaction between ascorbic acid and the reaction product, and that no action on the enzyme need be postulated. The fact that glutathione slowly decolorized the colored compound and also partially inhibited cytochrome oxidase (table 1) accounts for the greater inhibition reported by the above workers than was obtained in our experiments.

Thiouracil, sulfaguanidine, sulfathiazole and cysteine under the conditions used for testing did not inhibit cytochrome oxidase, and also these compounds did not decolorize the colored compound formed by oxidizing paraphenylenediamine with bromine. An explanation is not readily apparent for the lack of inhibition with thiouracil and sulfathiazole as compared to the inhibition reported by Paschkis et al for these compounds.

The succinic dehydrogenase activity of rat thyroid tissue was not inhibited by any of the compounds under the conditions of the experiments. In this connection it should be stated that the compounds tested for inhibition were added to the substrate at the time the tissue was added. Since cysteine did not inhibit under these conditions it is of interest to point out that Potter and DuBois (1943) using rat liver

TABLE 2. RESULTS SHOWING THAT ASCORBIC ACID AND GLUTATHIONE DECOLORIZE THE COMPOUND FORMED BY OXIDATION OF PARAPHENYLENEDIAMINE WITH BROMINE[1]

Compound	Bromine only	Glutathione	Ascorbic acid	Sulfathiasole	Sulfaguanidine	Cysteine	Thiourea	Thiouracil
Molarity of compound	Saturated aqueous solution	0.01	0.114	0.01	0.02	0.02	0.02	0.02
Amount of compound ml.	0.02	1	0.1	1	1	1	1	1
Colorimeter reading[2]	77	87.5	100	76.5	77.5	77.0	78.5	78.0

[1] Five ml. of phosphate buffer of pH 7 and 0.1 ionic strength was mixed in a test tube with 5 ml. of 0.2 per cent paraphenylenediamine. One of these tubes was kept, after addition of 1 ml. of water, for use as a blank control for the colorimeter readings. Two hundredths ml. of saturated aqueous bromine solution was added to each of the other tubes after which they were allowed to stand for 30 minutes at which time the compounds were added in the same concentration used in testing for cytochrome oxidase, the contents of the tubes were shaken, allowed to stand 10 minutes and read in the Evelyn photoelectric colorimeter.

[2] The colorimeter was set at 100 for the reading of the blank solution. The reading with bromine alone was 77, and a reading greater than 77 for any compound indicates that this compound decolorized the colored compound formed by bromine.

homogenate found that cysteine inhibited succinic dehydrogenase when addition of the substrate was delayed for 30 minutes, but there was no inhibition when the substrate was added at the beginning of the experiment. The latter is in agreement with the above result obtained with thyroid tissue. These workers also found that sulfanilamide did not inhibit succinic dehydrogenase even though the addition of substrate was delayed. It is also of interest that the succinic dehydrogenase activity of thyroid tissue of young rats was approximately double that found for thyroid tissue of adult rats (table 1).

Lerner and Chaikoff (1945) reported no difference in the total oxygen uptake of thyroid tissue in the absence and in the presence of certain sulfonamides, thiourea, or thiouracil and concluded that these compounds do not interfere with the cytochrome-cytochrome oxidase system of thyroid tissue. These results are in agreement with those of this report which indicate in addition that these compounds do not inhibit either enzyme of the succinoxidase system.

SUMMARY

Results obtained by use of ascorbic acid as substrate in the presence of cytochrome c show that sulfaguanidine, sulfathiazole, thiouracil and cysteine do not inhibit cytochrome oxidase of thyroid tissue, that thiourea causes little inhibition, and that glutathione inhibited approximately 50 per cent. Under the conditions used no inhibition of succinic dehydrogenase of thyroid tissue was obtained by use of these compounds. These results suggest that the sulfonamides tested do not act through the succinoxidase system.

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THE EFFECT OF ADRENALECTOMY ON THE ACCESSORY REPRODUCTIVE GLANDS OF MICE CASTRATED FOR SHORT PERIODS

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THE ISOLATION of a number of sterols from cortical extracts has left unanswered the question as to what extent these sterols are normally secreted by the adrenal. In the case of the androgens, isolated from adrenal extracts by Reichstein (1936) and others, it should be possible to evaluate the adrenal output quite directly, simply by comparing the state of androgen sensitive tissues in castrates and in adrenalectomized castrates. Such studies in rats (Howard '38, '41; Burrill and Greene '39, '40) have shown that for a brief period during normal development, the adrenal secretes sufficient androgen to cause an appreciable stimulation of the prostate. However, this effect does not occur after 50 days of age. Studies using transplanted prostates have shown that the age difference is not due to alterations in sensitivity of the tissue, but that there is less extra-gonadal androgen in adults (Price'41). Hence, andromimetic activity is not a normal function of the adult rat adrenal.

An exploration of the question in mice is of interest because in this species there is a clear cut adrenal histological response to prepuberal castration, namely the hypertrophy of the X zone. Furthermore, the presence of rather well developed seminal vesicle epithelium in young castrates having an adrenal X zone suggested that the X zone might be a source of androgens (Howard, 1939a). However, studies on the prostates and seminal vesicles of adrenalectomized castrates, which will now be described, seem to show that there is no physiologically appreciable androgen secretion by the mouse adrenal, even at the stage in which such a secretion does occur in the rat.

EXPERIMENTS

In view of the well known sensitivity to castration of adult seminal vesicle epithelium, the differentiation and prolonged maintenance of columnar epithelium in the vesicles of young castrated mice (Howard 1939a) merits special consideration. In contrast to this, some histological degeneration in the vesicles of adrenalectomized castrated

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mice was described in a preliminary note (Howard 1939b). However, clear cut regression was only observed in 9 out of 20 individuals found dead of adrenal insufficiency 6 to 13 days after operation. These animals were on a chow diet with 0.7% Nacl in their drinking water. All animals which were found dead did not show the regression, and it was not related to general autolysis. Four mice which were killed, 8 to 10 days after castration and adrenalectomy, in typical adrenal insufficiency, in no case showed vesicle regression. This suggested that the regression might be a terminal consequence of one of various sequences of metabolic events in adrenal insufficiency, rather than a reflection of a specific loss of adrenal androgens. This interpretation is born out by the present studies, in which the observations have been extended to mice of the H strain maintained in as normal a condition as possible after adrenalectomy. This was done by using a standard diet and a high Na:K ratio, or by giving 11-desoxycorticosterone acetate.

The possibility that desoxycorticosterone might be sufficiently androgenic to obscure an adrenal androgen effect in mice should be considered, in view of certain observations in the literature (Dantchakoff 1939, Hooker and Collins 1940). On the other hand, Greene and Burrill (1940) and Paschkis (1941) have found that it does not have an androgenic action in rats and chicks. Furthermore, in my studies on rats the use of desoxycorticosterone did not interfere with the demonstration of the adrenal androgen secretion.

A test was made of the effect of desoxycorticosterone in mice during the period in which the seminal vesicles are undergoing differentiation. Castration was performed on four mice at four days of age and two of these were given subcutaneous pellets of desoxycorticosterone. The animals were killed at 19 days of age, and prostates and vesicles examined histologically. No appreciable effect of the desoxycorticosterone was observed with this rather moderate treatment.

The effect of adrenalectomy, and desoxycorticosterone in adult castrated mice is also relevant in this connection. In adult mice, the regression of the vesicle epithelium following castration is not as rapid as in rats, but after 3 months the vesicles attain a state of fairly uniform degeneration. Eight mice were castrated at two months of age, and four of these were adrenalectomized and given subcutaneous pellets of desoxycorticosterone. After an interval of 3 months, during which all gained weight, the vesicles were examined. The vesicle epithelium had undergone a fairly uniform reduction to a cuboidal condition in both groups. The adrenalectomized mice on the sterol had a few areas of fairly well organized columnar epithelium, although the greater part of the vesicle was in a degenerate state with cuboidal epithelium. Thus we may conclude that there is no appreciably greater degree of castration atrophy in adrenalectomized castrated adults on desoxycorticosterone than in castrates having their adrenals. Further-

more, desoxycorticosterone pellets, which furnish an adequate maintenance treatment in mice, did not produce any androgenic effect great enough to interfere with the general picture of castration atrophy which occurs in adult mice.

A test was also made of the effect of injections in oil of larger quantities of desoxycorticosterone than are absorbed from pellets (see below). Eleven mice, weights 26.7 to 39.0 gm., were castrated, and after 10 days 6 were adrenalectomized and treated with injections of 2 mg. desoxycorticosterone acetate daily in peanut oil. One mg. was given intraperitoneally and one mg. subcutaneously. There was some leakage from subcutaneous injections. The castrated controls were given corresponding injections of oil, and all were killed after 10 days of treatment. The mean seminal vesicle body weight per cent ratios were: controls 0.102 and treated 0.108. Thus, no statistically significant androgenic effect of desoxycorticosterone was noted in terms of inhibition of vesicle size regression.

The studies on the younger X zone bearing mice will now be reported. The data from five series of observations are summarized in table II. In each series all mice were castrated, and half of the litter mate pairs were adrenalectomized.[1] The adrenalectomized mice were then treated either with desoxycorticosterone acetate or with salt therapy for various intervals. Those with desoxycorticosterone acetate pellets grew at approximately normal rates and appeared to be in good general condition. Some individuals on salt therapy died in insufficiency, but the majority were killed in an apparently normal state. The vesicles were removed by cutting them across at their point of emergence from the urogenital sinus just above the vas deferens. The prostate lobe adhering to the vesicle was not separated. Prostatic tissue may thus comprise about 20 per cent of the vesicle as weighed. Tissue was fixed in Bouin's fluid in the case of groups 1 to 4 while fresh and dry weights were obtained for group 5. The fixed tissue was washed for 1 to 2 hours in water, drained in a standard manner on filter paper, and weighed on a microbalance to 0.02 mg. The weights were reproducible to within 1 per cent. To obtain fresh weights, the tissues were placed on the wall of a stoppered vial containing a strip

[1] The technique of adrenalectomy in young mice has been previously discussed (Howard, 1937). A group of 18 three week old adrenalectomized mice, on a chow diet without added salt, all died within 12 days after the operation, most frequently at 4 days. A subsequent group of 67 three week old adrenalectomized animals on chow with 0.7% NaCl in their drinking water reacted as follows: 52% died within 13 days without increase in weight, 9% died within 31 days after showing subnormal growth, while the remaining 39% grew at an approximately normal rate and showed no gross signs of adrenal insufficiency. In a parallel group of 18 without NaCl in their drinking water, on the chow diet, 66% died within 14 days without increase in weight, and 34% showed moderate growth and no signs of insufficiency for two to four weeks. Hence this mode of NaCl therapy in mice appeared to be without appreciable ameliorative effect. Much better survival was obtained in the group on diet AD, discussed in the text. However, that group was older when adrenalectomized, and is not directly comparable to the groups maintained without therapy.

of filter paper moistened with Ringer's solution. The tissue was taken immediately to the weighing room, transferred to a stoppered weighing bottle which was kept in the balance case, and weighed to 0.02 mg. If the vesicles were returned to the vial and allowed to stand, they lost weight. In practice, the interval between dissection and weighing was uniform and as short as possible. The vesicles of young mice of 10 to 15 gm. body weight are capable of considerable increase in size when given an appropriate stimulus, such as prolan injections in intact mice (Callow and Deanesly, 1935). The maximum rate of vesicle enlargement under natural conditions occurs between 19 and 24 gms body weight. (Deanesly and Parkes, 1933).

TABLE I. COMPOSITION OF DIETS

Component	Diet A	Diet AD
Casein	12	25
Starch	39	31
Dextrin	26	10
Dried yeast	8	8
Wheat germ oil	1	1
Cod liver oil	1	1
Mazola oil	—	11
Salt mixture	3	4
Inert solids	10	9
Na:K mol ratio	1.84	3.33

The animals of group 1 were not weaned during the experiment, since nursing did not interfere with the detection of the androgenic phase of the adrenal in rats. The second group was on a diet of chow and tap water. In order to exclude the possibility that natural foods might contain materials which would influence the size of the castrated mice vesicles, and thus obscure a possible adrenal effect, groups 3 to 5 were maintained on standard diets described in Table I. In diet A the salt mixture was modified from Osborne and Mendel's IV (1913) by replacing K_2CO_3 by Na_2CO_3 to attain a Na/K ratio of 1.84. The mice on this diet were given in addition 0.9% NaCl in their drinking water. They were, however, allowed to nurse for a few days post-operatively. The salt mixture for diet AD, which had a Na/K ratio of 3.3, was made up as follows: NaCl, 10 mols; $MgSO_4$ 1; K_2HPO_4 1.5; $CaHPO_4$ 4; calcium lactate, 2; ferric citrate, 0.1; and trace elements as in Osborne and Mendel IV. With no additional NaCl in the drinking water, this proved to be the most satisfactory way of administering salt therapy which was tried. The group of 14 adrenalectomized mice maintained on the diet AD, with two exceptions which died 14 and 17 days post-operatively, were kept in fairly good condition for three to four weeks.

The castrates were paired with adrenalectomized castrates from the same litter. Minimal doses of desoxycorticosterone were used for one group in attempting to prolong life slightly while avoiding any

possibility of androgenic effects of excess sterol. If an adrenalectomized animal was found dead or in acute insufficiency, its vesicles were obtained with those of the castrate member of the pair, so that the interval between castration and weighing was the same for both members of any pair, although varying somewhat for different members of a group.

In table II it can be seen that their is little difference in any of the groups in the seminal vesicle body weight ratios. Such differences as occurred are not statistically significant. Various ages were used in order to test the possibility of a very brief period of an adrenal effect homologous to that observed in the rat, but no effect on the vesicles was detected. In the last group listed the vesicles were weighed

TABLE II. THE EFFECT OF ADRENALECTOMY ON THE SEMINAL VESICLES OF CASTRATED MICE[2]

Group	Age at castration (days)	Age at adrenalectomy (days)	Age at death (days)	Castrated				Adrenalectomized—castrated			
				No. of mice	Final body wt. (gms.)	Body wt. gain[5] (gms.)	Sem. ves. body wt. ratio $\times 10^5$	No. of mice	Final body wt. (gms.)	Body wt. gain[5] (gms.)	Sem. ves. body wt. ratio $\times 10^5$
1	14–16	14–16	22–23	7[n]	10.8	3.9	12.0	8[1,n]	11.0	3.3	11.8
2	20–21	20–21	26–27	9[a]	13.4	3.9	15.0	10[1,a]	14.0	4.2	13.7
3	20–22	24–26	30–34	7[3]	15.5	4.2	9.55	9[3]	14.1	2.6	7.42
4	23–24	23–24	33–38	6[4]	16.6	5.0	13.9	7[4]	13.8	2.4	15.0
5	20–23	40–45	56–68	15[4]	25.7	4.1	12.10	14[4]	23.0	1.1	12.26

[a] Diet: chow and tap water.
[n] Not weaned.
[1] Pellets of desoxycorticosterone acetate.
[2] Given 0.02 mg. per day desoxycorticosterone in oil intraperitoneally if daily weighing showed a loss of weight by the adrenalectomized member of the pair. On diet A, with 0.9% NaCl in drinking water.
[3] Given 0.4 cc. sterile 0.9% saline per day intraperitoneally beginning on fourth day after operation. On diet A, with 0.9% NaCl in drinking water.
[4] On diet AD.
[5] Body weight gain since time of adrenalectomy.

fresh, individually, then dried to constant weight at 110°, and weighed as a group. The mean fresh weights are given in the table. The corresponding figures for dried weights, per mouse, divided by the mean body weights, were for the castrated group, 2.95×10^{-5}, and for the adrenalectomized castrated, 3.01×10^{-5}. Since the castrated mice tend to have more depot fat than the adrenalectomized castrates, the ratios of the seminal vesicle weights to the body weights at castration were also calculated, and were for the castrates, 26.2×10^{-5}, ± 6.14 standard deviation, and for the adrenalectomized castrates, $23.5 \times 10^{-5} \pm 5.78$. Statistically the difference is not significant, and the figures show that their was no difference in vesicle size in relation to body weight at castration, as well as no difference relative to final body weight.

Histological studies on the first two groups of Table II did not reveal any evidence of an adrenal effect on the vesicles or on the ventral prostate of these animals. In groups three and four there were

[2] I am indebted to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, for supplies of 11-desoxycorticosterone acetate, and to Dr. R. S. Benua for assistance with the stand ard diets, and with some of the operations.

five adrenalectomized mice which were found dead. In these five individuals the seminal vesicles were in each case histologically less well developed than the litter mate castrated controls. The epithelium was cuboidal and the vesicles tended to be of a simple hollow structure without the highly developed convolutions found in the castrates. In some cases the nuclei were degenerating and the epithelium desquamating. However, those adrenalectomized individuals which did not die of adrenal insufficiency in groups three and four did not show any consistant alterations in the histological picture, in comparison with litter mate castrates. The epithelium was columnar, the alveolar walls profusely coiled, tending to fill up a large part of the vesicle contents without the very large lumina characteristic of the adult gland. Secretion granules were not present, the epithelium not being fully developed at this age.

The mice described in table II which were studied histologically, namely groups 1 to 4, consisted of age groups covering the period at which androgen secretion by the rat adrenal is at its maximum, as well as somewhat younger stages. These groups were, however, in most cases below the age at which the adrenal X zone attains its maximum size. Group 5 covers the period at which the X zone is at the heighth of its development, but the vesicles were used to obtain dry weights. A further series supplements group 5 for the purpose of histological study during the period of maximum X zone development, although the mice were maintained on desoxycorticosterone and were younger than group 5.

In this series thirty-nine mice were castrated and adrenalectomized, at three weeks of age, and treated with pellets of desoxycorticosterone acetate. The pellets were cylindrical, 2 and 3 mm. in diameter by about 2 to 3 mm. in length, and lost weight at the rate of 0.1 to 0.2 mg. per day per mouse. The pellets became encapsulated in a well vascularized connective tissue sheath. All animals were maintained in excellent general condition and grew at an approximately normal rate, doubling their body weight within twenty days after adrenalectomy.

The treated mice were killed after an interval of twenty days, and tissues prepared for microscopical examination. Nodules of tissue which were probably accessory adrenal cortex were observed in fourteen of the thirty-nine animals, and these were excluded from further study. Serial sections of the vesicles and prostates of the remaining twenty four adrenalectomized animals were examined systematically. Individual variation in the height of the columnar epithelium of the vesicles was apparent, but in general it was fairly well maintained, without secretion granules, in the condition typical of the X zone bearing castrated mice, which has been illustrated and described in detail elsewhere (Howard 1939a). Similarly, no effect of adrenalectomy on the various lobes of the prostate could be detected.

In groups 1 to 5 there were presumably a considerable number of individuals which retained accessory nodules of cortical tissue. Since, however, the amount of adrenal tissue was certainly greatly reduced, and probably insufficient to support life in over 50%, one might expect to detect some effect on the vesicles if the adrenals were physiologically significant as a source of androgens. In view of all the data, it appears that as long as a fatal collapse does not occur, the vesicle is not impaired by the absence of the adrenal. Hence the impairment of the vesicle which sometimes occurs in animals dying after adrenalectomy can hardly be due to a loss of adrenal androgens. It is conceivable that, in some sequence of metabolic events in adrenal insufficiency, the metabolism of the vesicle cells is so interfered with that degeneration occurs, either from non-androgenic causes, or from the failure of the production of androgens elsewhere in the body, perhaps in the genital tract itself.

The conclusion that there is no appreciable effect of adrenalectomy, at fourteen days of age and later, on the androgen sensitive tissues of recently castrated mice maintained in fair condition is thus based on histological study of the prostate and seminal vesicles, and on weight measurements of vesicles, both fixed weights, which are relatively reproducible, fresh weights, and dry weights. These observations are in agreement with the conclusions of Gersh and Grollman (1939) in regard to mice, and with the conclusions of McPhail (1944). Hence it appears reasonably certain that an androgenic adrenal secretion is not formed in normal mice of the strains and ages studied. The rat appears to be the only other species specifically tested as to this point, and here androgenic adrenal activity is exceedingly limited. Consequently, the assumption that the urinary androgens obtained from gonadectomized humans are of adrenal origin (Wintersteiner, 1941) should be viewed with some reserve. Urinary androgens are low in Addisons disease, but they are also low in other conditions (Friedgood, 1944).

The present findings should be viewed in relation to the interesting observations of Woolley and Little (1945), who noted that early castration of mice of the ce strain resulted, after 5 or more months, in the development of adrenal cortical carcinoma, associated in some cases with androgenic stimulation of accessory sex glands. Since strain differences might be involved, it would be interesting to know whether the adrenals of the ce strain produce appreciable androgens 3 to 6 weeks after castration. If, however, they do not, one might infer that the development of the adrenal function of secreting physiologically active androgens may actually be associated in adult mice with the development of the process of carcinogenesis in this tissue. Adrenal neoplasia in the human seems to be associated with abnormalities of steroid metabolism, and in certain types of adrenal tumor there is most probably an adrenal secretion of androgens (Pfiffner, 1942).

The interesting possibility remains that mice adrenals might normally produce androgens at still younger stages than those studied here, but in any case it may be concluded that the X zone is not a source of physiologically appreciable amounts of androgens.

The embryonic gonad apparently does not control organogenesis in the reproductive tract, (Moore 1944) and the period at which an androgenic sterol begins to influence morphogenesis is unknown. In the present study we are dealing with the maintenance of vesicle epithelium in a columnar rather than a cuboidal state. This specific character is maintained by gonad androgen in the adult, and hence it seems reasonable to inquite whether a similar substance is not involved at three to four weeks of age. Since both the gonad and the adrenal can be eliminated as unnecessary for vesicle maintenance at this stage, some other source of androgen seems to be suggested. The possibility of some degree of synthesis of sex hormones by extragonadal portions of the genital tract has not been excluded.

SUMMARY

Adrenalectomized mice were maintained on a standard diet with a Na:K ratio of 3.3, or on desoxycorticosterone. This sterol was not appreciably androgenic in mice. In adrenalectomized castrated mice, maintained in fair condition, there was no appreciable impairment of seminal vesicle weights, or of the histological state of the vesicles and prostates, compared with the level of maintainance in castrated controls, one to seven weeks after castration. In over half of all castrated individuals allowed to die of adrenal insufficiency, a clear cut histological regression of the vesicles was observed.

The failure to observe any impairment in the accesssory reproductive glands of adrenalectomized castrates maintained in fair condition, indicates that the mouse adrenal, even when the X zone is well developed, does not secrete physiologically appreciable amounts of androgens during some weeks after castration. Consequently the vesicle regression in animals found dead is probably not due to the loss of adrenal androgen.

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STUDIES ON THE "COLD TEST" AS A METHOD FOR THE ASSAY OF ADRENAL CORTICAL STEROIDS[1]

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In 1931, Hartman demonstrated a significant difference in the sensitivity of normal and adrenalectomized rats exposed to low environmental temperatures, and showed that the resistance of adrenalectomized rats can be increased by the administration of cortical extract. Since these observations were reported, numerous studies have been presented confirming these findings and suggesting the use of this technique as a quantitative method for the assay of adrenal cortical steroids. Selye and Schenker (1938) reported that a method involving the cold test could be utilized to detect as little as 0.05 cc. of Wilson's adrenal cortical extract. The quantitative aspects were not discussed in any detail by these authors and it is apparent that if the method is subjected to statistical analysis according to Wilson (1941) that the method as suggested by Selye and Schenker is statistically unreliable.

Weil and Browne (1939) and Venning, Hoffman and Browne (1944) have applied the Selye and Schenker method to the study of urinary cortin-like material. In the work of Venning, Hoffman and Browne (1944) the number of rats on each preparation was increased from 9 to 15 and the activity reported in terms of a "cold unit" (C.U.) which was defined as "the minimum amount of an extract or substance which when administered to a group of 15 adrenalectomized immature rats one half hour prior to their exposure to cold, will maintain at least two thirds of them in the active or inactive state at a time when at least two thirds of the controls are either comatose or dead." These latter workers administered the material in one dose before the animals were exposed to cold rather than in three doses as suggested by Selye and Schenker.

Both Dorfman, Horwitt and Fish (1942) and Vogt (1943) have

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used the cold test in rats to assay cortin-like material. These workers have used differences in mean survival times of treated and untreated adrenalectomized rats run simultaneously as the indicator of cortin-like activity. Roos (1943) has studied the cold test method in rats using the endpoint of drop in colonic temperatures rather than death. Kendal (1940) has employed a cold test method in rats to assay adrenal cortical extracts and pure adrenal steroids.

Zarrow (1942) studied the cold protecting properties of desoxycorticosterone acetate and progesterone in adrenalectomized mice. This author not only showed that these two steroids could protect adrenalectomized mice, but that the oily vehicle employed actually decreased the survival time of mice subjected to reduced temperatures.

This communication is concerned with studies on the cold test using both adrenalectomized rats and mice. An attempt has been made to apply a statistical analysis of the data to ascertain the usefulness of the method.

METHODS, MATERIALS, ANIMALS

White mice of unknown strain which were employed were of male sex except in one experiment as noted below. Albino male and female rats 22 to 24 days of age and weighing between 35 and 52 grams were used. The bulk of the rats were obtained from Sprague-Dawley, Madison, Wisconsin, and were bilaterally adrenalectomized the day after arrival A small minority, as designated later, were rats raised in this laboratory from Sprague-Dawley stock. The experiments were run between 12 and 24 hours after operation.

The animals, both mice and rats, were exposed to 5.5 ± 1.5 degrees C. for the duration of the experiment. Groups of 100 animals were usually run simultaneously, at least ten of which served as controls. During the course of the experiment, the animals were given no food or water and were kept in the dark except for a brief period of light every thirty minutes at which time observations were made. Each animal was housed in an individual wide mouth glass fruit jar containing a sheet of filter paper for bedding. In experiments requiring two injections during the course of the experiment, these injections were done in the cold room.

Oral administration was performed by means of a stomach tube immediately before the animals were placed in the cold room. Rats were unanesthetized but mice were subjected to light ether anaesthesia during this treatment. Rats received the total dose to be tested in 1 cc. of 10% ethanol while mice received 0.5 cc. of a similar solution. In some experiments on mice the material was dissolved in oil and injected subcutaneously.

Death was considered to have occurred when the animal had no visual signs of respiration, showed low body temperature, and on stimulation showed no responsive movements. This stage was easily discernable in the case of mice but more difficult in the case of rats.

The adrenal cortical extracts employed in these studies were purchased from the Wilson Laboratories and consisted of the ordinary commercial extract sold by this company. The 11-dehydrocorticosterone consisted of

crystalline material isolated from adrenal cortical extracts and the 11-desoxycorticosterone acetate was crystalline synthetic material.[2]

Extracts of men's urine were prepared with ethylene dichloride as a solvent and partial purification was accomplished by removal of inactive materials with alkali (Dorfman, Ross and Shipley, 1946).

STUDIES ON MICE

In order to study the survival rate after adrenalectomy, ninety-two male mice ranging in weight from 15 to 17 grams were bilaterally adrenalectomized and placed on a diet of oats and Purina dog chow. After 20 days only 83 per cent of the animals had died. The remaining

TABLE I. THE RESPONSE OF MALE MICE, EXPOSED TO LOW TEMPERATURES, TO PARENTERAL AND ORAL ADMINISTRATION OF CORTICAL EXTRACT[1]

Route of Administration	Weight of Animals Range gm.	Type of Mice	Substance Administered	Total Dose cc.	No. of Mice	Hours Mean Survival ±S. E.
		Sham Operated	Saline	0.3	20	10.4
Subcutaneously	15–18	Adrex	Saline	0.3	16	5.8
		Adrex	Cortical Extract	0.15	13	5.4
		Adrex	Cortical Extract	0.3	13	7.8
		Adrex	10% Alcohol	0.5	11	5.8 ±0.43
		Adrex	Cortical Extract	0.05	12	8.2 ±0.93
Orally	18–22	Adrex	Cortical Extract	0.1	10	7.0 ±0.85
		Adrex	Cortical Extract	0.2	11	8.5 ±1.14

[1] The animals treated parenterally were injected with ½ of the total dose at 0, 2 and 4 hours after placing in the cold room. Orally treated rats received the total dose at 0 hours.

16 animals were sacrificed and autopsy revealed no adrenal tissue in the region of the kidney either grossly or after histological study of suspicious tissue.

The comparative sensitivities of sham operated, adrenalectomized, and cortin-treated adrenalectomized mice to cold were studied. Male mice weighing between 15 and 18 grams were given varying doses of cortical extract twenty-four hours after adrenalectomy. It is evident from table I that the adrenalectomized mice were more sensitive to cold than sham operated animals and that the former were protected with 0.3 cc. of extract (+34%) but not with 0.15 cc. Oral administration gave significant protection at a dose level of 0.05 cc. but an increase of dose to 0.2 cc. did not materially increase the survival time. In another group of 22 mice half were pretreated with 0.4 cc. of extract divided into four injections, 20, 24, 28 and 40 hours postoperatively and placed in the cold room at 40 hours. These animals sur-

[2] We are indebted to Dr. E. C. Kendall of the Mayo Clinic and Dr. O. Kamm of Parke Davis and Co. for samples of 11-dehydrocorticosterone and to Ciba Pharmaceutical Products, Inc. for the 11-desoxycorticosterone acetate.

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vived an average of 6.0 ± 0.22 hours, as against 4.0 ± 0.26 hours for the half of the group which received similar doses of saline (50% increase).

The activity of desoxycorticosterone acetate in oil when administered by the latter technique is presented in table II. It is apparent that doses of 0.5 and 2.0 mg. were effective in protecting both male and female mice. In another experiment 15 mice received 0.4 cc. of corn oil and 15 served as uninjected controls. Those that recieved oil survived only 3.7 ± 0.2 hours as compared to 4.6 ± 0.35 hours for the controls. This finding confirms the report of Zarrow (1942) that an oily vehicle reduces the survival time of mice in the cold test. When desoxycorticosterone acetate in oil was given orally to a group of 10 mice a dose of 0.5 mg. produced a 40% increase in survival above controls ($p = 0.02$), but 2.0 mg. was ineffective.

TABLE II. COMPARATIVE REACTIVITY OF MALE AND FEMALE ADRENALECTOMIZED MICE (WT. 18–22 GM.) TO VARYING DOSES OF DESOXYCORTICOSTERONE ACETATE (INJECTED)

Number of Mice	Sex	Total Weight of Desoxycorticosterone Acetate mg.	Total Volume of Corn Oil cc.	Mean Survival Hours ± S.E.	Increase in Mean Survival %
35	M	0	0.4	4.6 ± 0.25	—
23	M	0.53	0.4	6.0 ± 0.60	30
13	M	2.0	0.4	6.7 ± 0.45	46
14	F	0	0.4	5.1 ± 0.29	—
16	F	0.53	0.4	6.3 ± 0.47	24
17	F	2.0	0.4	8.6 ± 0.76	69

STUDIES WITH RATS

In a preliminary experiment the survival rate of a group of rats after adrenalectomy was determined. Thirty-five male rats, 22 to 24 days of age and weighing 35 to 45 grams, were bilaterally adrenalectomized under ether anaesthesia and placed on a diet of Purina dog chow. Between the 4th and 12th day after operation all 35 animals died of adrenal insufficiency. The mean survival was 7.5 days.

The Effect of Various Factors on Survival Time of Untreated Rats in the Cold Test

The survival time of 14 sham operated rats was compared to that of a similar group of adrenalectomized rats. The adrenalectomized animals died between 4.0 and 7.5 hours (mean 5.8 ± 0.24), while the sham operated rats died between 8.0 and 33.5 hours (mean 15.9 ± 2.06) It was therefore confirmed that adrenal insufficiency *per se* is a major factor in the lethal outcome after exposure to cold.

The question as to the comparative survival of adrenalectomized rats treated by subcutaneous injection and stomach tube was important because of the different amounts of stress brought to bear on the

animals. The average survival time of 1085 male rats given 1 cc. of 10% ethanol by stomach tube was 5.75 ± 0.05 hours. This value did not differ significantly from that of a group of 102 rats receiving 0.6 cc. of saline by injection in which the average survival was 5.88 ± 0.12 hours.

Sex appeared to play no important part in survival. A group of 165 male rats raised in the laboratory survived an average of 5.56 ± 0.10 hours, while 79 similar female rats survived 5.41 ± 0.12 hours. In actual practice, however, animals chosen for assays were restricted to males to avoid possible variations incident to the estrus cycle.

In a series of 1085 male rats which had been used as control animals in assays performed by oral administration the survival time of

TABLE III. SURVIVAL OF CONTROL ADRENALECTOMIZED RATS RECEIVING 1 CC. OF 10% ETHANOL ORALLY

Mean Survival Hours	3.0 to 3.4	3.5 to 3.9	4.0 to 4.4	4.5 to 4.9	5.0 to 5.4	5.5 to 5.9	6.0 to 6.4	6.5 to 6.9	7.0 to 7.4	7.5 to 7.9
Number of Groups[1] in Given Limits	1	1	3	13	17	27	14	13	7	5
Range of Standard Error of Means	0.21	0.35	0.24 to 0.42	0.20 to 0.61	0.23 to 0.51	0.13 to 0.68	0.19 to 0.63	0.20 to 0.86	0.26 to 0.52	0.34 to 0.66

[1] The great majority of groups contained 10 animals. The number was rarely as low as 8 or as high as 20.

individual rats varied from 2.0 hours to 11.5 hours. The distribution curve for the series was normal and had a mode at 5.5–6.0 hours. The mean survival time of the groups of ten control rats employed during assays from time to time had a very wide spread. The extreme limits were 3.1 ± 0.21 to 7.9 ± 0.42 hours (table III). The difference between the above means has extremely high statistical significance and points to the presence of some factor aside from individual variability which operated to influence the survival of the various groups of animals. The data were therefore analyzed in an attempt to uncover such a factor.

In Figure I is illustrated the relationship between the weight of the animal and survival time. It is seen that with increasing body weight one can expect an increase in survival time, and that the relationship between these two variables is linear. In actual practice, however, the weight variable could not have been the cause of the wide spread in mean control survival. The *average* weight of animals in a given group varied only very slightly from group to group.

The effect of season of the year was studied. The average survival time of 625 control rats during the months from May to October was 5.41 hours, as compared to an average of 5.89 hours for 634 rats during

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the season from November to April. The difference, although significant, is small.

It was important to determine whether variations in the temperature of the cold room were of critical significance. Over a range from 3.3° to 6.7° C. a statistical analysis revealed no clear indication of any difference in mean survival of control groups.

Inasmuch as the rats which were received from Sprague-Dawley had undergone the stress incident to 2 days of transportation before

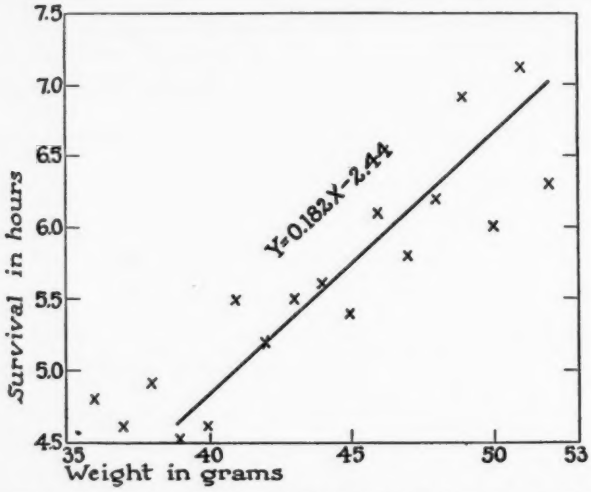

FIG. 1. The relationship of body weight to survival time of control rats is portrayed in the above graph. Two hundred thirty-four rats are represented. Each group contained an average of 14 rats.

arrival at the laboratory one might suspect that postoperative resistance would vary with the stress encountered during shipment. No evidence to support this suspicion was forthcoming. Thus, the survival time of 1085 rats shipped from the dealer was 5.75 ± 0.05 hours, and the average of 165 rats of the same strain raised in the laboratory was 5.56 ± 0.10 hours.

Within the range of 14 to 23 hours there was no influence of the post-operative time interval on the susceptibility of rats exposed to cold. In an experiment to test this question 17 animals placed in the cold room 23 hours after adrenalectomy survived 4.9 ± 0.55 hours. Sixteen animals placed in the room 14 hours after operation survived 4.8 ± 0.33 hours.

Responses Obtained with Cortical Hormones and Urine Extracts

The initial experiments were concerned with the response of animals to graded doses of adrenal cortical extract. The results, which

are presented in table IV, show that the animals were responsive to doses as low as 0.05 cc. and that the extract was effective both by oral route and subcutaneously. Cortical extract, however, was not adopted as a reference standard for assays because of its possible variable composition from lot to lot.

The question of the optimal number of animals in a group receiving a given dose of material was studied. The standard error of the mean was determined for groups of 5, 10, 15 and 20 rats which had been given enough hormone to produce significant responses. With groups above 10 the error did not improve sufficiently to warrant the extra expenditure of animals, time and material. Optimum group size for routine purposes was therefore accepted as 10 animals.

TABLE IV. THE COLD PROTECTING RESPONSE OF ADRENALECTOMIZED MALE RATS TO ADRENAL CORTICAL EXTRACT[1]

Subcutaneous Administration				Oral Administration			
Total Amount of Adrenal Cortical Extract Administered (cc.)	Number of Animals	Mean Survival Hours	Increase in Mean Survival %	Adrenal Cortical Extract Administered (cc.)	Number of Animals	Mean Survival Hours	Increase in Mean Survival %
0	21	5.4	—	0	20	6.0	—
0.06	21	6.9	28	0.05	18	9.3	55
0.15	21	7.6	41	0.10	18	9.7	62
0.30	20	8.8	63	0.20	18	11.8	97
0.60	20	10.3	91	0.40	19	11.8	97

[1] Subcutaneous injections were given at 0, 3 and 6 hours after placing in the Cold Room. Oral administration was at 0 hours.

Inasmuch as the body weight of individual rats affected their resistance to cold one might suspect that this factor would also influence sensitivity to cortical hormone. Such was actually found to be the case. An analysis of the survival times of 36 adrenalectomized rats receiving 0.05 cc. of cortical extract compared to 35 control rats revealed that treated rats averaging 40 gm. in weight lived no longer than controls. At 45 gm. of weight the treated rats survived 26% longer, at 50 gm. 44% longer, and at 55 gm., 60% longer. These estimates were obtained by the use of regression lines for control and treated rats derived by the method of least squares (Fisher, 1934).

A pooled sample of normal male urine was tested in graded doses administered orally. Assays of this sample were run on a number of successive days. The resulting curves are of considerable interest (Fig. 2). First of all it is apparent that activity may be demonstrated in very small amounts of urine (20 cc. or less). Secondly, it is apparent that the initial straight portion of the curve is very short. The useful range extends from about 15 cc. to 40 cc. of urine. A third characteristic is the failure of plateau formation. After reaching a peak the

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curves rapidly decline due, probably, to the complicating effect of toxic material in the extract. The final point to be noted is the very striking failure of response to any dose of extract on 9–20–44.

In connection with this last point, as work progressed, it soon became obvious that the sensitivity of the test animals from group to group was extremely inconstant. In Fig. 3 there are recorded responses on various occasions to varying doses of 11-dehydrocorticosterone

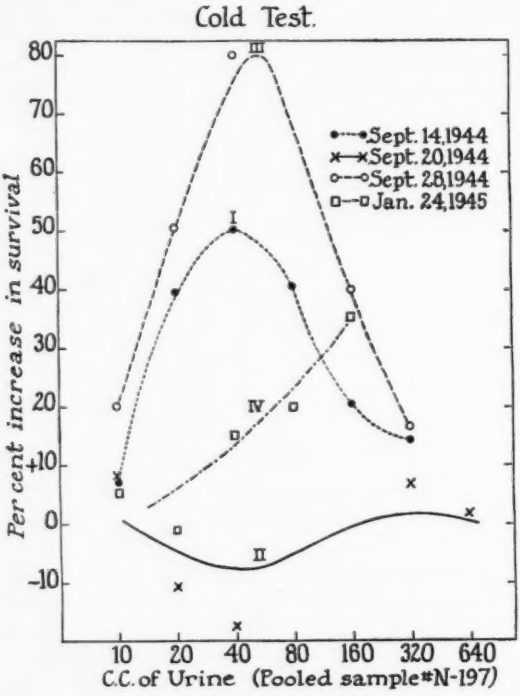

FIG. 2. The various responses were obtained with a pooled sample of normal male urine. Each point represents 10 animals. Percent increase is in terms of a control group of 10 animals.

(Comp. A of Kendall). Although a rising curve is discernible, the scatter of the various groups about the regression line is very marked. In the groups receiving 64 micrograms the prolongation of survival is significantly greater than the controls in about 50% of instances.

The possibility was considered that a correlation might exist between control survival time and sensitivity to hormone. This was tested statistically with the rats which received 64 micrograms of 11-dehydrocorticosterone, and in fact a slight tendency was found in the direction of increased sensitivity with decreased survival time of

control groups. The correlation was not good enough however, to explain the wide variations in sensitivity which were often encountered.

The Assay of Pooled Urine in Terms of 11-dehydrocorticosterone

In view of the wide scatter of data about the regression line in Fig. 3 an attempt was made to set up a standard assay curve after certain restrictions had been applied in the choice of acceptable observations. The first restriction consisted of the exclusion of all responses to the compound which were not significantly greater than

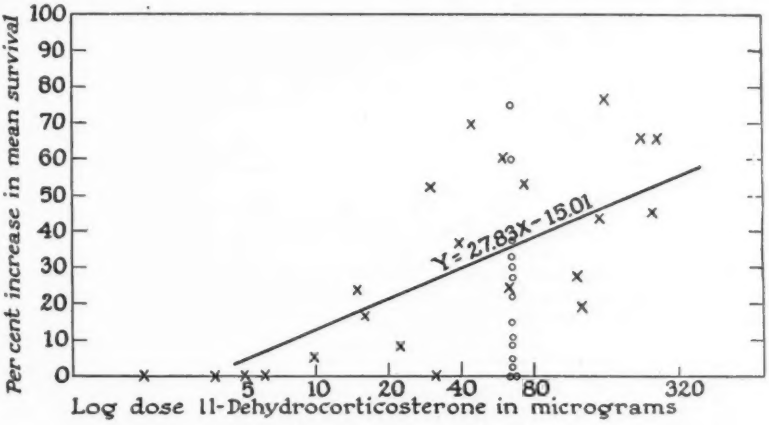

FIG. 3. The crosses indicate mean responses of groups containing 8 to 150 animals. In some instances the values represent composite means of several groups run on different occasions. The circles are groups of 10 animals which received 64 ug of dehydrocorticosterone on various occasions.

the controls (insensitive groups of animals). The second restriction consisted in the selection of data for construction of a standard curve from batches of animals where the average survival of control animals run with Comp. A approximated that of the control group of the particular lot of animals used for the urine extract. In the case of curves number I and III (Fig. 2) the control groups survived 4.6 and 3.9 hours respectively. The standard curve was constructed therefore, from data obtained when the controls were within the limits of 4.4 ± 0.37 hours. This curve along with those of the extract is illustrated in Fig. 4. In the case of curve IV (Fig. 2) the control group averaged 6.2 hours. The comparison with a corresponding standard curve (controls 5.7 ± 0.30 hours) is shown in Fig. 5. The compilation of results of these assays is found in table 5. The three estimates agree very closely and average 0.6 mg. of 11-dehydrocorticosterone per liter of urine.

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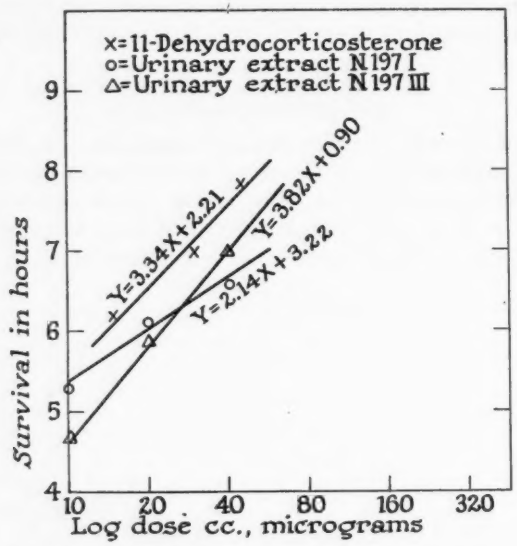

FIG. 4. The total number of animals used for the curve of 11-dehydrocorticosterone was 51. Each cross represents from 15 to 20 animals. The regression lines were determined by the method of least squares.

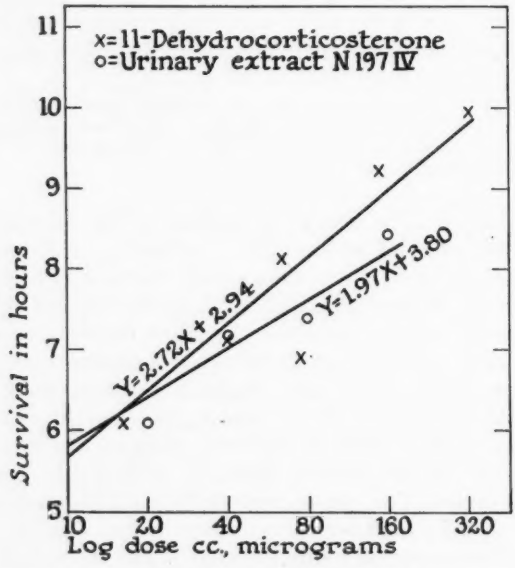

FIG. 5. The total number of animals used to determine the standard curve was 162. Each cross represents from 7 to 97 animals.

DISCUSSION

The studies which were made on the mouse were rather brief and were not pursued further because it appeared that this animal would not be as satisfactory as the rat for assay purposes. The failure of many mice to die promptly after adrenalectomy threw some doubt on the uniformity of production of typical adrenal insufficiency. It was also discouraging to find that rather large doses of hormone were required, that the gradation of response seemed short, and that the maximal response was of low magnitude.

A most extensive study was made on the cold test with the rat used as a test animal. Certain conclusions emerge from the accumu-

TABLE V. ASSAY OF POOLED URINARY EXTRACT IN TERMS OF 11-DEHYDROCORTICOSTERONE

Date	Experiment Number	Mg. of 11-Dehydro-corticosterone per Liter	Error Range[1] ($p=0.05$)	
			Per cent Low	Per cent High
9-14-44	I	0.60	50	102
9-28-44	III	0.66	40	67
1-24-45	IV	0.65	26	35

[1] The error of assay was calculated by the method of Bülbring (1938).

lated data. It may be said in favor of the cold test that it is simple, rapid, and potentially very sensitive. The one serious deficiency of the test, however, which mars its usefulness as a routine assay procedure, is the variability of sensitivity of animals from group to group. It was attempted to reduce this variability by restriction to male animals of a single strain, of identical age and average weight. Such variables as variation in the temperature of the cold room, post-operative time interval, season of the year, and transportation from the dealer were analyzed but the erratic behavior of the animals remained an enigma.

One variable which did prove to influence the sensitivity of animals was body weight. The higher sensitivity of larger animals does not necessarily imply that older animals would be preferable. Rats used in the experiments were of identical age. The superior state of nutrition of larger rats might minimize the lethal effects of cold due to nonspecific factors, and thereby bring out more sharply the life sustaining effect of specific therapy. It cannot be concluded that differences in weight caused the wide fluctuations in sensitivity between groups of animals. The mean weight of groups of animals did not vary in any great degree from time to time.

A practical question which arises is that of the applicability of the cold test for the assay of urinary cortin. If one should desire statistically reliable results with low limits of error it would appear that the

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method is impractical for routine use. Crystalline hormone is not available in sufficient quantity to run a standard curve for each specimen. Furthermore the chances are 1 in 2 that a group of animals will be sufficiently sensitive, and also tests may have to be repeated because of a failure to hit upon dosages of unknown that fall upon the short pre-toxic portion of the urine response curve. The assays of pooled urine reported herein show fairly good reliability but the results were attainable only because of the availability of accumulated data bearing on the response to 11-dehydrocorticosterone.

It is possible, however, to employ the test for rough estimates of urinary cortin content if a single dose of standard is run simultaneously with several unknowns. If two or more doses of unknown yield a rising curve of response, and a dose of 64 micrograms of dehydrocorticosterone produces a statistically significant ($p = 0.05$) increase in survival above the control group one may estimate the potency of the unknown. The latter is determined simply by noting which dose level on the curve of the unknown corresponds to the protecting effect of 64 micrograms of the standard. This method has been employed in a clinical study of cortin excretion (Shipley, Dorfman, Buchwald and Ross, 1946).

SUMMARY

The adrenalectomized mouse will respond to cortical extract and to desoxycorticosterone acetate when exposed to low environmental temperatures. From rather brief experiments it appears, however, that the mouse is not well adapted for assay purposes. The rat appears to be a more suitable animal in the cold test for the bioassay of urinary cortin.

The cold test is simple, rapid, and potentially very sensitive. Its usefulness is marred by an erratic variation in sensitivity of animals from group to group. The cause of this behavior has not been determined.

Rough estimates of urinary cortin may be made if one dose of standard is run simultaneously with several unknowns administered at 2 or more different dose levels.

The accurate assay of urinary cortin by the method requires large numbers of animals and quantities of pure hormone which make it impractical for extended routine use.

Sufficient data were obtained to show that a pooled sample of normal male urine contained the equivalent of 0.6 mg. of 11-dehydrocorticosterone per liter.

ACKNOWLEDGMENTS

We are indebted to Adeline S. Dorfman, Ethel Buchwald and Sara Johnston for their technical assistance.

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THE ASSAY OF ADRENAL CORTICAL MATERIAL BY MEANS OF A GLYCOGEN TEST IN THE ADRENALECTOMIZED MOUSE[1]

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THIS REPORT deals with an analysis of a method for assay of adrenal cortical material based on the deposition of glycogen in the liver of the fasting adrenalectomized mouse. The method was kindly communicated to us by Dr. K. Dobriner of the Memorial Hospital and was carried out almost exactly as originally described.

Reinecke and Kendall (1942) reported the use of glycogen deposition in the liver of the adrenalectomized rat as a method for the assay of adrenal cortical material. Although this method has many real advantages, as shown by these workers and later by Olson *et al.* (1944, 1944a), there still remained a need for a reproducible method sensitive enough for the assay of urinary cortin excretion under various conditions of health and disease. The method to be described and originally suggested by the Memorial Hospital group appears to satisfy such needs within certain limitations as shall be described in this communication.

METHOD

Male Swiss mice were maintained on a diet of Purina Dog Chow until operation. At a weight of 18 to 26 grams, they were anesthetized with ether and bilaterally adrenalectomized by the lumbar route. Immediately after operation each mouse was injected subcutaneously with 0.25 cc. of Upjohn's or Wilson's saline adrenal cortical extract. The operated mice were placed on the McCollum lactation diet (Agate and Zwemer, 1935) and were given 0.9% NaCl to drink.

On the morning of the fourth postoperative day the food was removed and the mice were injected seven times at hourly intervals. The total dose was always contained in 0.35 cc. of sesame oil, that is 0.05 cc. of oil per injection. One half hour after the last injection, the mice were weighed and

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[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and University Hospitals of Cleveland. Support was also received in the form of a grant from Sharp and Dohme.

anesthetized with nembutal. The entire liver was removed and placed in a 50 cc. graduated centrifuge tube containing 5 cc. of hot 30% potassium hydroxide. The tubes were placed in a boiling water bath and shaken at 5 minute intervals, and at the end of 20 minutes they were removed from the bath, 5.7 cc. of 95% ethanol were added, and the tubes were shaken and reheated in the water bath. The alcoholic solution was allowed to come to a boil, the tubes were removed from the water bath and cooled in cold water. After centrifugation at high speed for ten minutes, the supernatent fluid was decanted off and the tubes inverted on a piece of paper towelling. They were dried in this manner overnight.

The following day 5 cc. of NH_2SO_4 were added to each tube. The tubes were covered with tear bulbs and placed in a boiling water bath for 2 to 2½ hours. After hydrolysis of the glycogen the tubes were removed from the bath and cooled. Two drops of phenol red indicator were added and the contents were neutralized with *N* NaOH. If the volume was below 10 cc. it was adjusted to 10 cc. with distilled water. Total reducing substances in 5 cc. of the neutralized diluted solutions were then determined by the methods of Somogyi (1937) and Shaffer and Somogyi (1933). The total liver glycogen was calculated and referred to the body weight of the mouse so that the responses were expressed as mg. of glycogen per 10 grams of body weight.

Urinary Extract. The urine was collected with toluene as preservative and extracted within 48 hours of collection. Extraction of untreated urine was effected with one quarter volume of ethylene dichloride by shaking for two minutes on a mechanical shaking machine. The layers were separated with a Sharples centrifuge. This procedure was repeated twice more. The combined ethylene dichloride extracts were evaporated to dryness in vacuo and the residue taken up in chloroform, 50 cc per liter of original urine. The chloroform solution was extracted with 0.1 *N* sodium hydroxide and the neutral fraction prepared according to the method described by Venning, Hoffman, and Browne (1944). The residue containing the neutral fraction was dissolved in 95% ethanol and stored at −15° C. until used. Under these conditions the urinary cortin extract appears to be stable for at least four months.

Adrenal Cortical Extract in Oil. This was a commercial extract prepared from pig adrenal by the Upjohn Company and labeled as containing 2 mg. of 17-hydroxy-11-dehydrocorticosterone equivalent per cc. by a survival growth method of assay.

11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone. The crystalline samples of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone were kindly supplied to us by Dr. E. C. Kendall of the Mayo Clinic and Dr. O. Kamm of Parke Davis and Company.

EXPERIMENTAL

Tables I and II contain the glycogen deposition responses to 11-dehydrocorticosterone (compound A Kendall) and 17-hydroxy-11-dehydrocorticosterone (compound E Kendall) respectively. Both experimental groups are compared to a group of 71 control mice which were treated the same as the experimental mice including the administration of the requisite amount of sesame oil. Tables III and IV

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TABLE I. GLYCOGEN DEPOSITION IN THE ADRENALECTOMIZED MALE MOUSE TREATED WITH 11-DEHYDROCORTICOSTERONE (COMPOUND A KENDALL)

Number of Mice	11-Dehydrocorticosterone Administered Micrograms	Glycogen in Liver Mg./10 gm. of Body Weight Mean ±S.E.
71	0	0.75 ±0.16
11	20	0.21 ±0.09
9	40	2.53 ±0.90
14	80	3.03 ±0.86
11	160	5.06 ±0.99
11	320	7.92 ±0.67

TABLE II. GLYCOGEN DEPOSITION IN THE ADRENALECTOMIZED MALE MOUSE TREATED WITH 17-HYDROXY-11-DEHYDROCORTICOSTERONE (COMPOUND E KENDALL)

Number of Mice	17-Hydroxy-11-dehydro-corticosterone Administered Micrograms	Glycogen in Liver Mg./10 gm. of Body Weight Mean ±S.E.
71	0	0.75 ±0.16
8	5	1.14 ±1.00
9	10	2.09 ±0.78
16	20	2.13 ±0.40
12	40	6.96 ±1.08
9	80	15.90 ±2.21

TABLE III. GLYCOGEN DEPOSITION IN THE ADRENALECTOMIZED MALE MOUSE TREATED WITH URINARY EXTRACT

Number of Mice	Urinary Extract Equivalent Administered cc.	Glycogen in Liver Mg./10 gm. of Body Weight Mean ±S.E.
71	0	0.75 ±0.16
12	20	0.16 ±0.06
10	40	0.29 ±0.10
19	80	2.08 ±0.64
17	160	3.20 ±0.98
11	320	3.90 ±1.55
6	640	9.75 ±2.56

TABLE IV. GLYCOGEN DEPOSITION IN THE ADRENALECTOMIZED MALE MOUSE TREATED WITH PIG ADRENAL CORTICAL EXTRACT

Number of Mice	Pig Adrenal Cortical Extract cc.	Glycogen in Liver Mg./10 gm. of Body Weight Mean
71	0.00	0.75
21	0.01	2.30
20	0.02	4.08
21	0.04	5.76
21	0.08	6.52
21	0.16	19.24

TABLE V. THE LINEAR REGRESSION FORMULAE OF 11-DEHYDROCORTICOSTERONE, 17-HYDROXY-11-DEHYDROCORTICOSTERONE, URINARY EXTRACT, AND PIG ADRENAL CORTICAL EXTRACT USING THE RELATIONSHIP BETWEEN LOGARITHM OF THE DOSE AND THE RESPONSE

Material Administered	Linear Regression Formulae	Remarks
11-Dehydrocorticosterone	$Y=6.38x-8.15$[1] $Y=3.54\pm1.09+6.46\pm0.81(x-\bar{x})$	
17-Hydroxy-11-dehydrocorticosterone	$Y=22.23x-27.36$ $Y=7.05\pm0.74+22.23\pm3.08(x-\bar{x})$	From 3 points, 20, 40 and 80 micrograms
	$Y=6.66x-4.81$	From 4 points, 5, 10, 20 and 40 micrograms
Urinary Extract	$Y=4.88x-7.80$ $Y=1.96\pm1.09+4.88\pm0.72(x-\bar{x})$	
Pig Adrenal Cortical	$Y=4.79x+2.38$	From 4 points, 0.01, 0.02, 0.04 and 0.08 cc. $x=\log 100\times$dose

[1] This includes 137 observations not included in Table I.

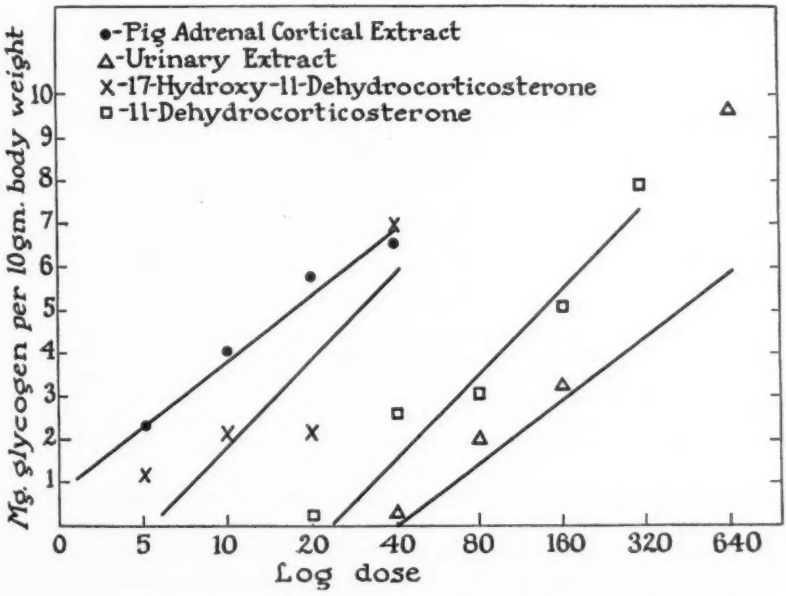

FIG. 1. Liver glycogen responses to increasing doses of pig adrenal cortical extract, urinary extract, 17-hydroxy-11-dehydrocorticosterone, and 11-dehydrocorticosterone. Doses of pig adrenal cortical extract are plotted as the logarithm of 500 times the dose in cc. of oil solution. The urinary extract is plotted as the logarithm of the dose in cc. and the doses of the two adrenal cortical steroids are represented as the logarithm of the dose in micrograms.

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contain the increasing responses with increasing doses of an extract derived from normal men's urine and pig adrenal cortical extract respectively. Since the bulk of the data on the pig adrenal cortical extract was derived from the analysis of pooled samples of livers it is not possible to give the standard errors of the means.

Table V is a summary of the linear regression formulae of 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, urinary

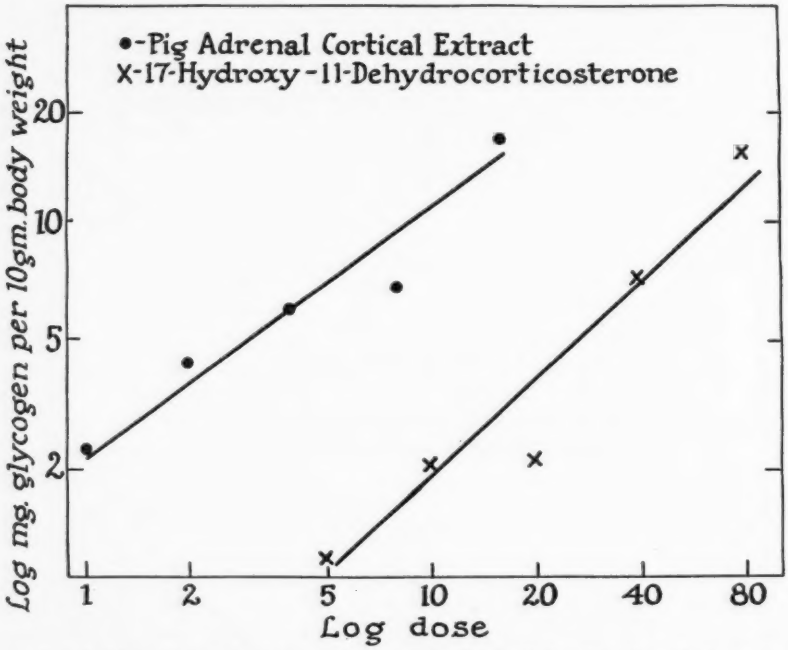

FIG. 2. Liver glycogen responses to increasing doses of pig adrenal cortical extract and 17-hydroxy-11-dehydrocorticosterone. Doses of pig adrenal cortical extract are plotted as the logarithm of 100 times the dose in cc. of oil solution while doses of 17-hydroxy-11-dehydrocorticosterone are expressed as the logarithm of the weight in micrograms. The formula for 17-hydroxy-11-dehydrocorticosterone was $Y=1.317\ x-0.961$ and the formula for the pig adrenal cortical extract was $Y=0.659\ x+0.370$.

extract and pig adrenal cortical extract using the relationship between the logarithm of the dose and the response for the lower portion of the curve. The similarity of the slopes of the curves of urinary extract, 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone (range 5 to 40 micrograms), and pig adrenal cortical extract (range 0.01 to 0.08 cc.) is represented in Figure 1. When the significancy of difference between slopes of urinary extract and either 11-dehydrocorticosterone or 17-hydroxy-11-dehydrocorticosterone (4 points from 5 to 40 micrograms) are calculated according to the method of Fisher

(1934) no significant differences are found. Since the slope of the pig adrenal cortical extract is so close to that of the urinary extract, that is, 4.88 for the urinary extract as compared to 4.79 for the adrenal cortical extract, it may be assumed that the slope of the tissue extract is not significantly different from the slopes of the two crystalline compounds.

If all five experimentally determined points are plotted as a log dose-response relationship for both 17-hydroxy-11-dehydrocorticos-

TABLE VI. VARIATIONS IN GROUP MEANS OF CONTROLS

Number of Mice	Mg. Glycogen per 10 g. Body Weight
71	0.75 ± 0.16
3	0.04
3	0.15
3	0.17
4	0.49
4	0.75
5	0.19
5	0.41
5	0.47
5	2.02
6	0.08
6	0.09
6	0.10
6	0.11
6	0.21
6	0.37
6	0.55
6	1.34
6	1.66
6	1.72
6	2.30
6	2.46
6	2.94
7	0.38
7	1.48
7	1.97
9	2.47
10	0.28
10	0.32
10	0.76
12	1.69
12	2.30
13	0.11
13	3.15
17	0.38

terone and pig adrenal cortical extract it is seen that the curves assume an exponential character. If these curves are reconsidered as an exponential function and plotted as the relationship between the logarithm of the dose and the logarithm of the response a linear relationship becomes more apparent. These characteristics are illustrated in Figure 2.

Table VI represents the values of liver glycogen in milligrams of glycogen per 10 grams of body weight for the control animals. These control mice received a total dose of 0.35 cc. of sesame oil and were

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treated exactly as the experimental animals. A group of 71 animals which were run individually had a mean of 0.75 ± 0.16. The remaining values in the table illustrate the large variations that can be expected. The mean of the remaining 242 control mice was 1.08 which is probably not significantly different from the mean of the first group of 71 mice.

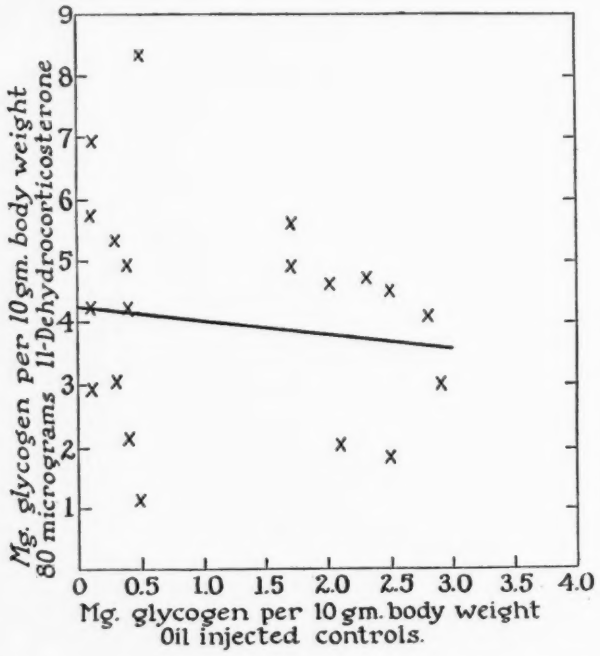

FIG. 3. Variation in liver glycogen response to 80 micrograms of 11-dehydrocorticosterone with respect to oil injected controls run simultaneously. Each point represents approximately 7 to 8 hormone injected mice and 7 to 8 oil injected controls. There are a total of 153 oil injected control mice and 142 11-dehydrocorticosterone treated mice represented.

In order to obtain evidence as to whether the sensitivity of mice could be correlated with variations in original glycogen levels, a comparison was made of responses of groups to a standard 80 microgram dose of 11-dehydrocorticosterone when varying control glycogen levels were encountered in groups run simultaneously. It is seen from Figure 3 that no apparent relationship exists. This is further evident from the fact that the calculated slope is -0.2. When the control value approaches 0 a value of 4.2 would be found for 80 micrograms of compound A while at the other extreme a control value of 3.0 would give a value of 3.6 mg. for the compound A response. This would result in

TABLE VII. VARIATIONS IN RESPONSE TO 80 MICROGRAMS OF 11-DEHYDROCORTICOSTERONE

(Values read from standard 11-dehydrocorticosterone curve. Approximately 5 mice per group.)

Number of Mice	Amount Found Micrograms	Errors %
3	113	+ 41
4	49	− 39
4	103	+ 29
4	105	+ 31
5	425	+431
5	100	+ 25
5	63	− 21
5	265	+230
6	65	− 19
6	130	+ 63
6	70	− 13
6	70	− 13

a maximum deviation of −10 to +8 micrograms of 11-dehydrocorticosterone.

Twelve experiments were run involving the administration of 80 micrograms of 11-dehydrocorticosterone to groups of animals containing approximately 5 mice per group (from 3 to 6) (Table VII). The response in milligrams of glycogen per 10 grams of body weight was referred to a 11-dehydrocorticosterone standard curve (56 animals) and the error of determination calculated. In 11 of the 12 experiments the error ranged from 41 per cent low to 230 per cent high. In one experiment the error was 431 per cent high. The predictable error calculated by the method of Bülbring (1938) is actually −72 to +260 per cent when $p = 0.05$.

In Table VIII a similar sort of analysis is presented, except that

TABLE VIII. VARIATIONS IN RESPONSE TO 80 MICROGRAMS OF 11-DEHYDROCORTICOSTERONE

(Values read from standard 11-dehydrocorticosterone curve. Approximately 10 mice per group.)

Number of Mice	Amount Found Micrograms	Errors %
8	200	+150
8	40	− 50
8	49	− 39
10	44	− 45
10	78	− 3
10	130	+ 63
10	170	+113
10	130	+ 63
10	163	+138
10	160	+100
10	81	+ 1
11	118	+ 48
11	123	+ 54
11	83	+ 38
12	49	− 39
14	60	− 25

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in these experiments approximately 10 mice were used (from 8 to 14). Here errors ranging from −50 to +150 per cent were found in 16 experiments. The predictable error is actually −59 to +150 per cent when $p = 0.05$.

DISCUSSION AND CONCLUSIONS

From the experiences presented in this paper it appears that the method as devised by the Memorial Hospital group can be used for the assay of relatively small amounts of cortin-like material. Evaluation of the suitability of the method may be considered under these general groupings: (1) relationship between respective slopes of reference standard and urinary cortin-like material; (2) values of control levels; and (3) expected errors of assay under a given set of conditions. The data presented in this communication indicate some of the trends peculiar to this assay procedure.

When one considers a log-dose-response relationship for the urinary extract, 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, and pig adrenal cortical extract at responses up to 8 mg. of glycogen per 10 grams of body weight, linear relationships result with slopes which do not appear to vary significantly from each other. The significancy of differences in slopes was calculated, according to the method of Fisher (1934), for urinary extract and 11-dehydrocorticosterone; urinary extract and 17-hydroxy-11-dehydrocorticosterone; and for 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone. In each case no statistically significant difference in slope could be demonstrated. In the case of the pig adrenal cortical extract the bulk of data was derived from determinations of glycogen on pools of livers thus making it impossible to calculate variations. If we assume the variation to be of the same magnitude as that found in the study of the urinary extract and crystalline adrenal cortical compounds it seems reasonable to conclude that the slope of 4.79 for the pig adrenal cortical extract is not significantly different from the slopes of urinary extract (4.88), 11-dehydrocorticosterone (6.38) and 17-hydroxy-11-dehydrocorticosterone (6.66). Therefore, in the assay of urinary cortin-like material any of the other three materials should serve as adequate reference materials. In actual studies which have been made on concentrations of urinary cortin-like material 11-dehydrocorticosterone has served as the reference standard.

With respect to two materials, pig adrenal cortical extract and 17-hydroxy-11-dehydrocorticosterone, the responses were studied up to values of 19.24 and 15.9 mg. of glycogen per 10 gm. of body weight respectively. If all the data on these materials are considered we find an exponential relationship which when plotted as logarithm dose against logarithm response appears to be linear. Whether this would hold for the urinary extract and 11-dehydrocorticosterone is not known at this time. However, this extension of assay range may not

be of value for the assay of urinary extract due to the large amount of urine that would be required. This additional range from an approximately 8 mg. response to approximately 16 mg. may be of value for the assay of glandular extract and crystalline adrenal cortical steroids.

The study on the control levels indicates a rather wide variation. When groups of mice containing 3 to 17 animals were run the mean response varied from 0.04 mg. to 3.15 mg. of glycogen per 10 grams of body weight. Of the 34 experiments two resulted in means greater than 2.5 mg. and seven resulted in means greater than 2.0. Thus, one must exercise caution in interpreting assay values below 2.5 mg.

Since the control values did show rather wide fluctuations in means it was of interest to see if the sensitivity of simultaneously injected animals varied accordingly. However, no relationship between control means and intensity of response could be demonstrated when a standard dose of 80 micrograms of 11-dehydrocorticosterone was run in parallel with control animals. This comparison was made 20 times.

The expected error of assay when $p = 0.05$ and using 5 animals at one point within the assay range was found to be -72 to $+260$ per cent. In actual practice it was shown that when approximately 5 mice were employed in 11 of 12 trials the error ranged from -41 to $+230$. In one instance the error exceeded the predicted value and was $+431$ per cent. The predicted error for 10 mice per group, when $p = 0.05$, was -59 to $+150$ per cent as compared to the results of 16 trials in which approximately 10 mice were used and an error range of -50 to $+150$ per cent was found.

The potency ratio of the urinary extract to 11-dehydrocorticosterone has been calculated by the method of Irwin (1937). It was found that 1 microgram of 11-dehydrocorticosterone is equivalent to 2.4 cc. of the urinary extract or one liter of urine is equivalent to 417 micrograms of 11-dehydrocorticosterone. The error of this estimate calculated by the method of Bülbring (1938) indicates that when $p = 0.05$ the error may be 46% higher or 33% lower than the reported value.

SUMMARY

A study of the feasibility of assaying urinary cortin-like material by a glocogen deposition method in adrenalectomized mice, originally devised by the Memorial Hospital group, has been presented.

It has been found that within a certain defined range the relationship between log dose and response is linear and the slopes similar for urinary extract, 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, and a pig adrenal cortical extract. It may be concluded further that a urinary extract may be assayed in terms of either of the two crystalline adrenal cortical steroids or in terms of the pig adrenal cortical extract. The use of 5 mice at one point within the assay range at $p = 0.05$ may result in an error of -72 to $+260$ per

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cent while the use of 10 mice under similar conditions may result in an error of −59 to +150. Although control groups of mice showed rather large variations in mean glycogen values no significant correlation between the magnitude of the control level and the response of simultaneously injected animals to a standard dose of hormone could be demonstrated.

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THE RELATIVE POTENCIES OF ADRENAL CORTICAL STEROIDS AS DETERMINED BY A COLD PROTECTION TEST AND BY A GLYCOGEN DEPOSITION TEST[1,2]

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THIS COMMUNICATION is concerned with the comparative activities of 11-desoxycorticosterone acetate, corticosterone, 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone on a cold protection test and the comparative activities of 17-hydroxy-11-dehydrocorticosterone and 11-dehydrocorticosterone on a test involving the deposition of glycogen in the fasting adrenalectomized male mouse.

MATERIALS AND METHODS

The cold protection test involved the use of 22 to 24 day old male albino rats of the Sprague-Dawley strain. They were bilaterally adrenalectomized 24 to 48 hours after arrival in the laboratory and fed Purina Dog Chow before and after adrenalectomy. After oral administration of the compound the animals were exposed to $5.5° \pm 1.5°$ C. without food and water (12 to 24 hours after operation). Observations were made at ½ hour intervals until death. At least ten animals composed each group receiving a given dose of hormone and 10 animals received only solvent so as to serve as controls. A description of the method and analysis of some variables involved has been presented elsewhere (Dorfman, Shipley, Schiller, Horwitt, 1946).

The cold test was employed in two ways. For some comparisons standard conditions were selected in which all the groups used for the analysis were chosen so that the mean survival of the various control groups which were run in parallel with the treated groups, did not differ significantly from the control group means run at the time the standard curve was determined. For other calculations the 2 compounds under consideration were run simultaneously at various dose levels.

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[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and University Hospitals of Cleveland. Support was also received from Sharp and Dohme Inc.

[2] These studies were made possible by the generous contributions of 17-hydroxy-11-dehydrocorticosterone, 11-dehydrocorticosterone, and corticosterone from Dr. E. C. Kendall; 17-hydroxy-11-dehydrocorticosterone from Parke Davis and Co. and 11-desoxycorticosterone acetate from Ciba Pharmaceutical Products Inc.

The glycogen assay method employed has been published (Dobriner *et al.* 1946; Dorfman, Ross, and Shipley, 1946). The method involved the use of adrenalectomized male mice.

STATISTICAL METHODS

Regression coefficients were calculated according to Fisher (1934). The relative potencies were calculated by the method of Irwin (1937) and the error of the potency ratios was calculated by the method of Bülbring (1938).

EXPERIMENTAL

Comparisons of Compounds on the Basis of the Cold Test

Figure 1 illustrated the responses of increasing doses of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone. The doses are represented in log of micrograms and the response as hours

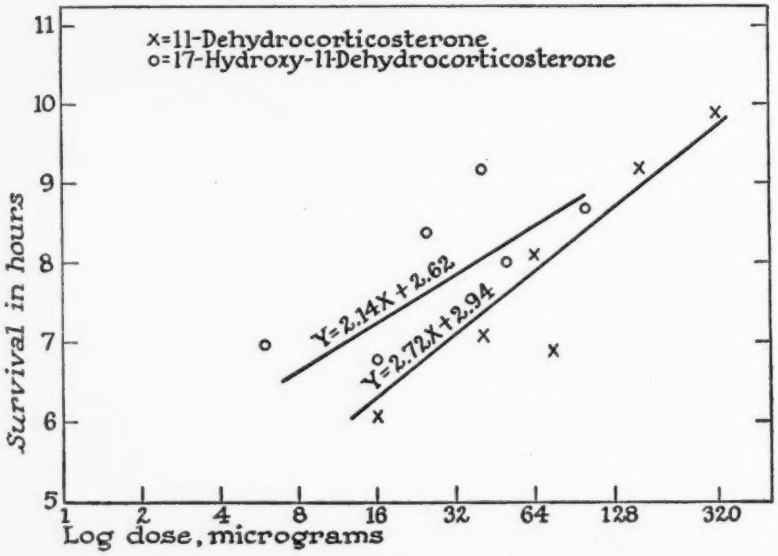

FIG. 1. Log dose-response curves for 11-dehydrocorticosterone (162 rats) and 17-hydroxy-11-dehydrocorticosterone (113 rats).
Control mean survival was 5.7 hours.

of survival of the various groups. It was found that the slopes of 11-dehydrocoticosterone and 17-hydroxy-11-dehydrocorticosterone were 2.72 and 2.14 respectively which were not significantly different (Fisher, 1934). These slopes were determined from assays where the control group means did not differ significantly from 5.7 hours. The curve for 11-dehydrocorticosterone represented observations on 162 animals while the curve for 17-hydroxy-11-dehydrocorticosterone represented 113 observations. When these two curves were compared it was found that one microgram of 11-dehydrocorticosterone was

equivalent to 0.5 microgram of 17-hydroxy-11-dehydrocorticosterone with an error of −32 per cent and +46 per cent (Table I). Three other comparisons of the potencies of these two compounds were made. In two instances these adrenal cortical steroids were assayed simultaneously and in one instance comparisons of relative potencies were made when the control group means did not differ significantly from 7.3 hours. In the first run where the two hormones were run simultaneously, it was found that one microgram of 11-dehydrocorticosterone was equivalent to 0.2 micrograms of 17-hydroxy-11-dehydrocorticosterone while in the second run the value was 0.3. In these latter

TABLE I. THE RELATIVE POTENCIES OF VARIOUS ADRENAL CORTICAL STEROIDS ON THE BASIS OF A COLD PROTECTION TEST

Compound	Total Number of Rats	Amount of Compound Equivalent to One Microgram of 11-Dehydrocorticosterone	Error of Estimate $P=0.05$		Remarks
			Low %	High %	
17-Hydroxy-11-Dehydrocorticosterone	275	0.5	32	46	
	159	0.3	49	93	
	76	0.2	48	92	Compounds run simultaneously
	76	0.3	48	92	Compounds run simultaneously
Corticosterone	238	3.8	35	55	
11-Desoxy-Corticosterone Acetate	192	4.0	42	71	
	135	4.7	54	115	

comparisons the error of the potency ratios were rather high, −48 per cent and +92 per cent.

Comparisons of the activity of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone when the mean survival of the controls for both compounds were 7.3 showed a potency of 0.3 micrograms of 17-hydroxy-11-dehydrocorticosterone equal to one microgram of 11-dehydrocorticosterone. This comparison was calculated on the basis of 159 observations (Table I).

Figure 2 illustrates the comparative responses of 11-dehydrocorticosterone and corticosterone. The slope of 11-dehydrocorticosterone was 2.72 as compared to the slope of corticosterone which was 2.14. The difference in slopes was not significant. As indicated in Table I it was found that one microgram of 11-dehydrocorticosterone was equivalent to 3.8 micrograms of corticosterone. The error of the estimate was found to be −35 per cent to +55 per cent.

11-desoxycorticosterone acetate was compared to 11-dehydrocorticosterone on the basis of the cold test at control levels of both 5.7 and 7.3 hours. The respective slopes were not significantly different at either control level of mean survival. The potency as related to 11-dehydrocorticosterone is represented in Table I.

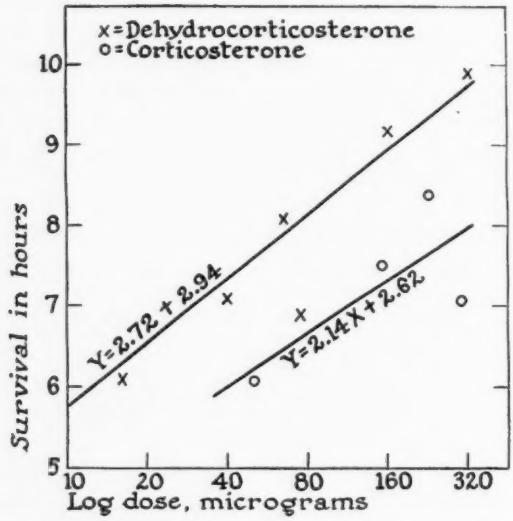

FIG. 2. Log dose-response curves for 11-dehydrocorticosterone (162 rats) and corticosterone (76 rats).
Control mean survival was 5.7 hours.

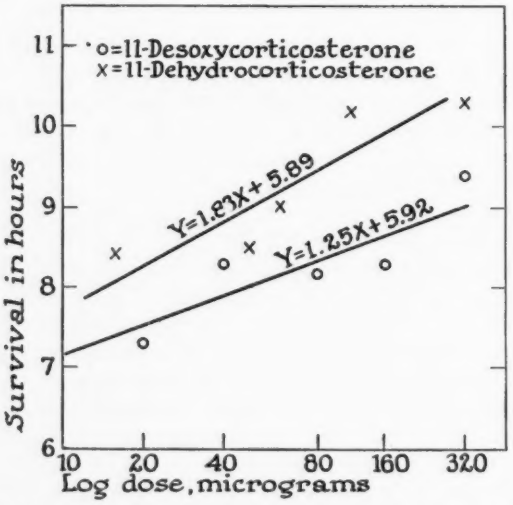

FIG. 3. Log dose-response curves for 11-dehydrocorticosterone (83 rats) and 11-desoxycorticosterone acetate (52 rats).
Control mean survival was 7.3 hours.

Figure 3 compares the slopes of 11-desoxycorticosterone acetate and 11-dehydrocorticosterone when the respective control means were 7.3 hours. 11-desoxycorticosterone acetate was found to be approximately one-fourth as active as 11-dehydrocorticosterone and of about the same order of activity as that found for corticosterone. The actual values found for the two comparisons were one microgram of 11-dehydrocorticosterone equal to 4.0 and 4.7 micrograms of 11-desoxycorticosterone acetate respectively.

Comparisons of Compounds and a Pig Adrenal Cortical Extract by a Glycogen Deposition Test

Table II tabulates the various regression formulae for 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone and a pig adrenal cortical extract using a glycogen deposition test in adrenalectomized mice. Essentially two types of linear relationships have been calculated, one using log dose-response over a limited range (up to 8 mg. of glycogen per 10 grams of body weight), and the other a log

TABLE II. REGRESSION FORMULAE OF VARIOUS COMPOUNDS AND EXTRACTS ON BASIS OF GLYCOGEN ASSAY

Compound	Number of Points	Number of Mice	Regression Formulae	Remarks
11-Dehydrocorticosterone	5	193	$Y=6.38x+8.15$	Log x
17-Hydroxy-11-Dehydrocorticosterone	4	45	$Y=6.66x-4.81$	Log x
	5	54	$Y=1.317x-0.961$	Log x − Log y
Pig Adrenal Cortical Extract	4	83	$Y=4.79x+2.38$	Log x x = log 100 times the dose in cc.
	5	104	$Y=0.659x+0.37$	Log x − Log y x = log 100 times the dose in cc.

dose-log response relationship which includes responses up to 20 mg. of glycogen per 10 grams of body weight. More details on these responses have been reported in another communication (Dorfman, Ross, and Shipley, 1946).

Table III presents the comparison of the potencies of 17-hydroxy-11-dehydrocorticosterone and a pig adrenal cortical extract to that of 11-dehydrocorticosterone. It was found that 0.26 micrograms of 17-hydroxy-11-dehydrocorticosterone was equivalent to one microgram of 11-dehydrocorticosterone. It was found also that 0.00028 cc. of the pig adrenal cortical extract was equivalent to one microgram of 11-dehydrocorticosterone.

The relative potencies of 17-hydroxy-11-dehydrocorticosterone and pig adrenal cortical extract when calculated from the 4 point log dose-response and log dose-log response relationships is tabulated

TABLE III. COMPARATIVE POTENCIES OF VARIOUS ADRENAL CORTICAL COMPOUNDS AND EXTRACTS ON BASIS OF A GLYCOGEN DEPOSITION TEST

Compound	Amount Equivalent to One Microgram of 11-Dehydrocorticosterone		Error of Estimate $P=0.05$	
	μgm.	cc.	Low %	High −%
17-Hydroxy-11-Dehydrocorticosterone (4 pts.)	0.26	—	25	33
Pig Adrenal Cortical Extract	—	0.00028	23	30

in Table IV. No significant difference in relative potencies is seen when the two methods are compared. Thus by the log dose-response relationship it was found that one cc. of the pig adrenal cortical extract contained the equivalent of 995 micrograms of 17-hydroxy-11-dehydrocorticosterone while from the log dose-log response relationship we find a value of 776 micrograms per cc. of extract.

TABLE IV. RELATIVE POTENCIES OF PIG ADRENAL CORTICAL EXTRACT AND 17-HYDROXY-11-DEHYDROCORTICOSTERONE ON THE GLYCOGEN TEST

Amount of 17-Hydroxy-11-Dehydrocorticosterone Equivalent to 1 cc. of Pig Adrenal Cortical Extract (micrograms)	Error of Estimate $P=0.05$		Relationship Used
	Low %	High %	
995	32	46	4 points for each log dose-response
776	30	43	5 points for each log dose-log response

DISCUSSION AND CONCLUSIONS

In Table V a summary of the comparative potencies of the various adrenal cortical steroids on the basis of both the cold test and the glycogen test is presented. If the activity of 17-hydroxy-11-dehydrocorticosterone is set at 100, it is seen that 11-dehydrocorticosterone is 26% as active on the basis of the glycogen assay. This appears to be at variance with the results of Olson, Thayer, and Kopp (1944) who

TABLE V. RELATIVE POTENCIES OF ADRENAL CORTICAL STEROIDS ON THE BASIS OF A COLD PROTECTION TEST AND A GLYCOGEN TEST EXPRESSED AS THE PERCENTAGE OF THE ACTIVITY OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE

Compound	Cold Test %	Glycogen Test %
17-Hydroxy-11-Dehydrocorticosterone	100	100
11-Dehydrocorticosterone	33	26
Corticosterone	8.7	—
11-Desoxycorticosterone Acetate	7.6	<2

reported approximately equal activity for 17-hydroxy-11-dehydrocorticosterone and 11-dehydrocorticosterone on the basis of a liver glycogen test in the fasting adrenalectomized rat. A possible explanation for this discrepancy may be the fact that these latter workers used a method which involves the building up of glycogen whereas in the method employed in this work the hormone essentially protects the fall of liver glycogen. It must be further remembered that in one instance the rat was employed while in the other the mouse served as the test animal.

On the basis of the cold test 17-hydroxy-11-dehydrocorticosterone was again found to be the most active (Table V). 11-dehydrocorticosterone was found to be 33% as active as the latter hormone while corticosterone and 11-desoxycorticosterone acetate were found to be 8.7 and 7.6% as active respectively. Venning, Hoffman, and Browne (1944) have reported equal activities for corticosterone and 17-hydroxy-11-dehydrocorticosterone. These workers also reported that desoxycorticosterone acetate was approximately 1% as active as either corticosterone or 17-hydroxy-11-dehydrocorticosterone. Kendall (1941) has reported that 17-hydroxy-11-dehydrocorticosterone is approximately twice as active as corticosterone on the basis of a cold test.

The question as to the activity of the pig adrenal cortical extract on the basis of the glycogen assay is of some interest. This extract (#8120-6) prepared by the Upjohn Company and sold under the label of Lipo-adrenal cortex was labeled as being equivalent in biological activity to approximately 2 mg. of 17-hydroxy-11-dehydrocorticosterone or 4 mg. of corticosterone per cc. when assayed by a Survival-Growth method. The comparative assays of the extract and 17-hydroxy-11-dehydrocorticosterone on the basis of a glycogen assay reported here indicates that 1 cc. of the extract contains the equivalent in biological activity to 886 micrograms of 17-hydroxy-11-dehydrocorticosterone. This value is approximately one half the activity found by the survival-growth method.

SUMMARY

On the basis of a cold protection test employing adrenalectomized immature male rats the relative potencies of four adrenal cortical steroids was determined. It was found that 17-hydroxy-11-dehydrocorticosterone was the most active of the substances studied and that 11-dehydrocorticosterone, corticosterone, and 11-desoxycorticosterone acetate were respectively 1/3, 1/12, and 1/13 as active.

On the basis of a liver glycogen test using male adrenalectomized mice it was found that 11-dehydrocorticosterone was 1/4 as active as 17-hydroxy-11-dehydrocorticosterone.

Data are also included on the assay of a pig adrenal cortical extract.

ACKNOWLEDGMENTS

We are indebted to Adeline S. Dorfman, Ethel Buchwald and Sara Johnston for their technical assistance.

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QUANTITATIVE METHODS FOR THE BIO-ASSAY OF THE GLYCOGENIC ACTIVITY OF STEROIDS AND URINARY EXTRACTS[1]

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THIS PROJECT was undertaken to develop a sensitive and specific method for the quantitative bio-assay of urinary extracts with respect to their glycogenic activity. (Dobriner, 1944, 1945; Eggleston, 1944.) The presence of such activity in these extracts was demonstrated by Venning, Hoffman and Browne, (1942). Reinecke and Kendall (1942) reported a method, to be called method A, for the assay of adrenal cortical extracts based on their glycogenic action in fasted, adrenalectomized rats. This method has been extensively studied by Olsen *et al.* (1944). In this laboratory it was found that to obtain a significant increase over the fasting liver glycogen level relatively large amounts of cortical hormones or urinary extracts were necessary. A method that would yield positive results with the least possible quantity of material was therefore sought. The use of mice in place of rats did not increase the sensitivity sufficiently. The three procedures which finally were evolved are based on the concept that adrenal cortical hormones repress the breakdown of liver glycogen in adrenalectomized animals (Britton, 1932). The first, the "titration method," to be referred to as method B, is used for the quantitative determination of glycogenic activity in urinary extracts, while the second, or "priming method," to be called method C, is used to determine the presence or absence of activity in very small amounts of pure compounds. Both methods will be described. As they are identical in most respects, they will be presented together and differences will be noted where they occur.

Shorr (1945) has suggested that more consistent results might be obtained if total fermentable sugars rather than liver glycogen were measured. Errors from glycogenolysis, occasionally caused by struggling of the animals or delay in immersing the livers in KOH,

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should be markedly reduced. A third method was therefore devised and will be referred to as D, "the sugar method."

METHODS

I. *Choice and Preparation of Animals.*

Male mice of the Swiss or Rockland strain weighing 18–24 gms. were adrenalectomized bilaterally under sodium pentobarbital anesthesia. Upon awakening, each animal was given a subcutaneous injection of 0.25 ml. of Upjohn's aqueous adrenal cortical extract and placed in a cage in a draft-free, constant temperature room at 78° F. Normal saline was provided for drinking purposes and the animals were placed on a modified McCollum's lactation diet (Agate and Zwemer, 1935), containing 0.04 per cent of potassium, to which 10 per cent of sucrose and 1 per cent of cod liver oil have been added. Care was taken that food was always available to the animals.

An attempt was made to maintain the animals, both rats and mice, on Purina Dog Chow, but less than 50 per cent of each species survived for as long as 10 days after adrenalectomy. It was thought that the potassium content of this diet (0.7 per cent) was too high. Therefore, McCollum's lactation diet with only 0.04 per cent of potassium was substituted. Under ordinary laboratory conditions, the survival rate for four days was 90 to 100 per cent in a series of several hundred mice, but during extreme changes of temperature the rate of survival dropped to between 45 and 75 per cent. When the animals were kept in the constant temperature room at 78° F. the percentage of survival was maintained at 90 to 100 per cent. It is of interest that 84 per cent of the test group of mice can survive for ten days on a diet containing 1.4 per cent of potassium if they were kept in the constant temperature room.

Inasmuch as mice were found to be more resistant than rats to the trauma of operation and anesthesia, were easier to handle for injection, and deposited more glycogen with smaller quantities of cortical steroids, they were considered to be the animal of choice. Male mice were selected to avoid any possible effect of the estrus cycle.

The general condition of the mice was improved when they were given an injection of 0.25 ml. of Upjohn's adrenal cortical extract when they regained consciousness after adrenalectomy. This was therefore adopted as a routine procedure.

II. *Experimental Procedure.*

Reinecke-Kendall Method (A) (1942). This method used adrenalectomized rats which had been fasted for sixteen hours prior to the injection period.

Titration method (B). On the fourth day after adrenalectomy, the hourly injection of the substance to be tested was begun. At the time of the first injection, the animals were transferred to clean cages provided with saline but no food. Throughout the entire period of injections, the animals were kept in the constant temperature room at 78° F.

Priming method (C). This differed from (B) in two respects only. 1. At 4:30 P.M. on the fourth day following adrenalectomy, the animals were injected with 0.4 ml. of Upjohn's adrenal cortical extract from a fresh ampule. 2. The injection of the material under investigation was then

started at 8:30 on the following morning and carried through as outlined under (B).

Sugar method (D). This differed from (B) in that Carworth Farms CF_1 strain mice were used and the total fermentable sugar was determined instead of total liver glycogen.

Subsequent Procedures Applicable to Methods B and C

Beginning exactly one half hour after the last injection of the first animal of a series, the animals were weighed and killed, by snapping their necks, in the order in which they were injected. Within 30 seconds after each mouse was killed, its liver was removed and dropped into 5.0 ml. of hot 30 per cent potassium hydroxide in a graduated 50 ml. centrifuge tube which had been standing covered in a boiling water bath.

Liver glycogen was determined by the method of Good, Kramer & Somogyi (1933) with the sugar reagent of Shaffer-Hartman and Somogyi #2 (Peters and Van Slyke 1932). The glycogen was calculated as glucose and recorded as mg. per 10 gm. of body weight of the mouse.

Subsequent Procedure for Sugar Method (D)

The animals were killed as in methods B and C. Immediately upon removal each liver was dropped into a graduated 50 ml. Pyrex centrifuge tube containing 10 ml. of N H_2SO_4. The liver was then digested for three hours in a boiling water bath, with frequent and vigorous agitation. After digestion the tube was allowed to cool and 0.8 ml. of 40 per cent NaOH was added, followed by sufficient N NaOH to bring the solution to the first pink color with phenolphthalein. The proteins were precipitated by the method of Somogyi (1930). Sugar analyses were done on the supernatant fluid by the method described under B and C.

To determine the non-fermentable reducing substances, 5 ml. of the supernatant were pipetted into a graduated 15 ml. centrifuge tube containing 5 ml. of washed and centrifuged Fleischmann's baker's yeast. The suspension was thoroughly mixed and allowed to stand at room temperature for one hour. The tube was then centrifuged and 2 ml. of the supernatant fluid were taken for determination.[2]

The total fermentable sugar was calculated by subtracting the non-fermentable reducing substances from the total reducing substances. These results also were reported as mg. of total fermentable sugar per 10 gm. of mouse.

III. *Material.*

The crystalline steroid or preparation from urinary sources, (Eggleston, 1946) to be assayed was dried at temperatures not exceeding 50° C. in a 50 ml. Florence flask. Then 0.2 ml. of absolute alcohol was added and mixed with the dried material by rotation. After thirty minutes oil was added as described below. After mixing, the oil was allowed to stand in contact with the extract overnight or longer in the cold. The flask was then rotated for a

[2] We are indebted to Dr. Robert F. Furchgott of Cornell University Medical College for his advice in the technical details of the blood sugar methods of Somogyi as applied to tissues.

few seconds in warm water and inverted in a rack over a graduated 15 ml. centrifuge tube into which the solution was permitted to drain for at least five hours, but not longer than overnight.

To determine the volume of oil in which the sample was to be dissolved, the following scheme was used. Each mouse will receive seven injections of 0.05 ml. each, making a total of 0.35 ml. Urinary extracts were titrated to an end point, three mice being used for each level. One-eighth of a 48-hour sample or the equivalent of six hours of urine will be injected into each of three animals for the first test so the sample must be dissolved in 8×0.35 ml. or 2.8 ml. of oil. Injecting three mice will take 1.05 ml. and 1.75 ml. will remain for dilution.

IV. *Titration—method B*

The titration was carried out in the following manner: A total of 0.35

TABLE 1. TYPICAL PROTOCOL OF A URINARY EXTRACT TITRATION

Titration Method, (B).

1. An average liver glycogen of 1.0 mg. per 10 gm. of mouse was found to be produced by 2μg. of Compound E.
2. Twenty-four hour sample of urine was extracted.
3. Extract was taken up in 6×0.35 cc. of oil.
4. Of this 0.35 cc. were injected into each of three mice: therefore each mouse received the equivalent of 4 hours of urine. This was positive. Therefore 4 hours + (sample A).
5. Sample (A) diluted 1:1 — 2 hours per mouse still positive (B)
6. Sample (B) diluted 1:1 — 1 hour per mouse still positive (C)
7. Sample (C) diluted 1:4 — ½ hour per mouse still positive (D)
8. Sample (D) diluted 1:1 — ¼ hour per mouse negative (E)
9. 1½×E inj. per mouse — ⅛ hour per mouse borderline (F)

Hours:	4	2	1	½	¼	⅛
Samples:	A	B	C	D	E	F
	5.9	10.3	6.4	2.8	0.3	2.1
	9.3	1.8	2.5	2.4	0.2	0.2
	12.9	6.8	10.0	0.9	0.3	1.1
Average:	9.3	6.3	6.3	2.0	0.3	1.1
Result:	+	+	+	+	−	±

Therefore ½ hour is equivalent to 2 μg. of Compound E, or equal 1 m.u., 1 hour = 12 m.u., or 24 hours = 288 m.u. Activity of sample is not less than 192 m.u. per 24 hours, and not more than 384 m.u. per 24 hours.

ml. of the extract in oil was injected into each of three mice over the seven-hour period. If the average of the liver glycogens was greater than 0.7–1.3 mg. per 10 gm. of mouse, the urinary extract was diluted 1:1 with sesame oil and the test repeated. Further dilutions, with testing at each level were continued until the average of the liver glycogens of three animals fell between 0.7 and 1.3 mg. per 10 gm. of mouse. This was considered to be the end point or the minimal positive response. As a check a final dilution of the test material, amounting to less than 1:1 was prepared and tested. This should have given less than 0.5 mg. of glycogen per 10 gm. of mouse. If more than 0.5 mg. was found, it was assumed that the animals given the next larger amount were hyporeactive. Additional dilutions were then made until negative results were obtained. The protocol of an assay is outlined in detail in Table 1. If a more delicate end point was desired, larger doses of the extracts might be given, up to a total of 0.7 ml. in one injection period.

V. *Standardization.*

When pure compounds of unknown activity were to be tested, 5–10 mg. were dissolved in enough oil for injection into five or ten mice. If very high glycogen values were obtained the assay was repeated with a smaller amount of material. For pure compounds tested with method C one half the number of animals used was injected with the unknown substance and the other half received the standard. The potency of the compound was then read from a standard curve, Figure 1.

When method D was used the test was set up in the same manner as

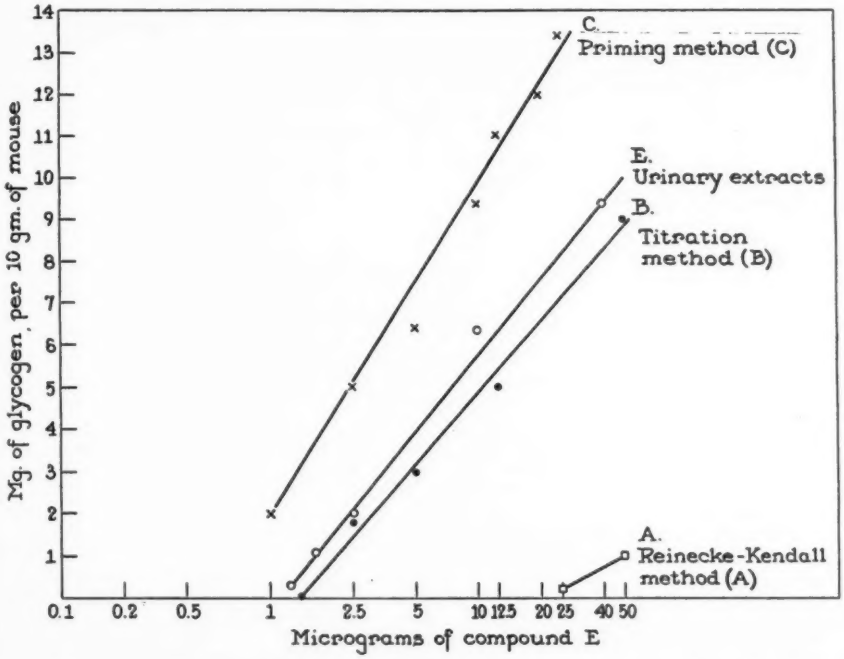

FIG. 1. Dose-response curves for methods A, B, and C. And a curve for urinary extracts E.

method C and the results were read from the standard curve, Fig. 2. The number of animals to be injected with the unknown and the standard depends on the precision of results desired as determined from Table 2.

The compound 17-hydroxy-11-dehydrocorticosterone[3] (Kendall's Compound E) was chosen as the reference standard because it is a pure crystalline compound with high glycogenic activity (Ingle, 1942). It was found that 2μg. of Compound E when tested by method B produced an average liver glycogen of 1.0 mg. per 10 gm. mouse (Fig. 1). The number of hours of urine collection represented by the extract necessary to produce a response of 0.7–1.3 mg. of glycogen per 10 gm. of mouse was therefore equivalent to 2μg. of

[3] We are grateful to Dr. E. C. Kendall for supplying us with the Compound E used in this work.

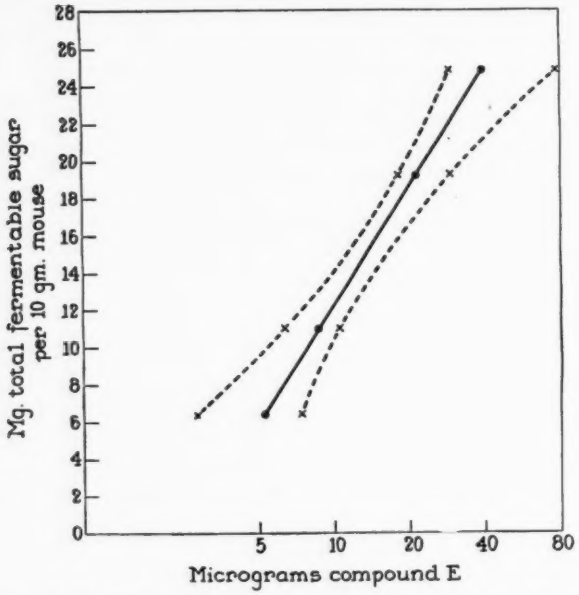

FIG. 2. Regression of response on log dose with exact confidence limits at .05 points for estimated most probable log dose, in method D.

TABLE 2. CONFIDENCE LIMITS OF A POTENCY RATIO IN TERMS OF PERCENTAGE OF ITSELF FOR THE GLYCOGEN METHOD, (C) AND THE SUGAR METHOD (D)

Animals on each dose.	Glycogen method, (C).			Sugar method, (D).		
	Low	Potency ratio	High	Low	Potency ratio	High
5				66%	1.0	152%
10	37%	1.0	267%	76%	1.0	131%
20	51%	1.0	195%	83%	1.0	121%
50	66%	1.0	152%	89%	1.0	112%

Compound E. From this, the activity of urinary extracts was calculated in terms of mouse glycogen units (m.u.) per 24 hours where 1 mouse glycogen unit was defined as the glycogenic activity equivalent to that produced by one microgram of Compound E.

These three procedures were compared with method A, that of Reinecke and Kendall (1942).

RESULTS

I. *Liver glycogen levels at the start of the experimental period.*

The liver glycogen levels at the time of the first injection were determined for each of the first three methods under consideration. The results are shown in figure 3.

II. *Liver glycogen at the end of the experimental period.*

In order to establish the control levels in two of the methods under discussion (B & C), the liver glycogen contents at the end of the seven-hour injection periods were determined when sesame oil alone was used as the test substance. These glycogen levels were found to be identical with those obtained after the total 24-hour fast in the

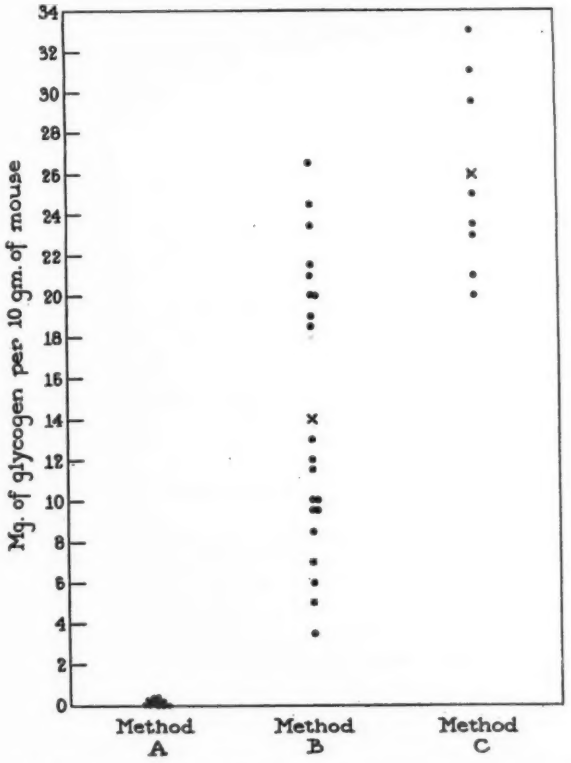

FIG. 3. Liver glycogen levels at the beginning of the injection period in methods A, B, and C.
• = individual values
x = average values

Reinecke-Kendall test (A), namely, less than 0.5 mg. per 10 gm. of mouse. At the end of six hours, or one hour before the last injection, the values had already fallen to insignificant levels. (See Fig. 4). This insured a margin of safety in both methods in the event that the test substance was inactive since adequate time had been allowed for the liver glycogen to become depleted.

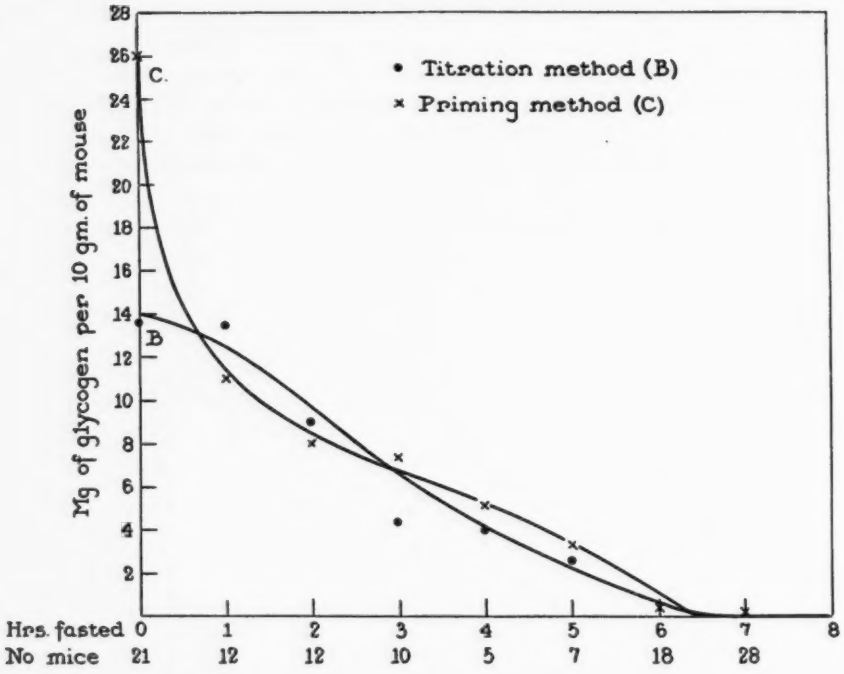

FIG. 4. Decrease in liver glycogen levels during the seven hourfast in methods B and C.

TABLE 3. GLYCOGEN AND FERMENTABLE SUGAR RESULTS IN NORMAL AND ADRENALECTOMIZED MICE MAINTAINED UNDER IDENTICAL CONDITIONS

	Mg. Glycogen per 10 gm. of mouse.					Mg. Fermentable Sugar per 10 gm. of mouse.				
	Range		Mean	High/Low		Range		Mean	High/Low
	Low	High				Low	High		
NORMAL MALE MICE										
A_1	3.9	12.5	7.1	3.2	B_1	4.3	12.6	8.4	2.9
A_2	2.0	8.2	5.0	4.1	B_2	3.2	9.2	7.3	2.9
A_3	1.9 *	5.0	3.7	2.6	B_3	6.7 **	14.3	9.9	2.1
ADRENALECTOMIZED MALE MICE										
A_4	2.0	5.8	3.4	2.9	B_4	2.9	6.0	5.1	2.1
A_5	0.6	7.3	3.3	12.2	B_5	1.4	10.6	5.1	7.6#
A_6	2.8 ***	11.4	7.6	4.1	B_6	11.8 ****	24.6	17.1	2.1

* 32 animals, ** 33 animals, *** 31 animals, **** 25 animals. # This figure is widely out of line, but the reason was not determinable. A and B animals with corresponding subscripts were treated identically except that glycogen determinations were made on the livers of the A animals, and sugar determinations on the livers of the B Animals.

III. *Controls for the sugar method, D.*

Three groups of approximately twenty normal male mice each, maintained on a modified McCollum's lactation diet (Agate & Zwemer, 1935), were divided into two equal subgroups, A and B. The A animals were used for glycogen determinations and the B animals were used for total fermentable sugar analyses. From Table 3 it is seen that the mean total sugar values are consistently higher than the mean glycogen values. The ratios of the maximum to the minimum values obtained in any one series were more uniform and smaller when total sugar determinations were made.

A series of fifty-six male mice were similarly treated, four days after adrenalectomy. Similar results were obtained (Table 3).

IV. *Liver glycogen curves with Compound E.*

The relationship between the amount of Compound E injected and the resultant liver glycogen was studied. Fig. 1 shows the dose-

TABLE 4. RESPONSES TO DIFFERENT DOSES OF COMPOUND E IN METHODS C AND D

Micrograms of Compound E.	Method D. Mgs. of fermentable sugar per 10 gms. of mouse.	Method C. Mgs. of glycogen per 10 gms. of mouse.
0	2.8	0.7 ± 0.06
0.5		1.6 ± 0.03
1.0		2.0 ± 0.08
2.5		5.0 ± 0.7
5.0	6.4 ± 0.4	6.0 ± 0.6
10.0	11.0 ± 0.2	9.3 ± 1.0
12.5		11.1 ± 1.5
20.0	19.2 ± 0.8	12.0 ± 1.0
25.0		13.3 ± 0.7
40.0	24.8 ± 1.0	

response curves for the first three methods under consideration. The standard curve (A) for method A was established with thirty-seven mice. (B) is the dose-response curve for method B which was compiled from observations on forty mice. (C) is the standard curve for method C based on results in two hundred and eight animals. The standard deviations are given in Table 4. This curve, with a standard deviation of 3.7, and a slope of $8.4 + 8.1\ (X\text{-}\bar{x})$, is approximately parallel to (B). (B) and (C) are both linear when plotted on semilogarithmic paper, and (C) was tested and found to satisfy statistical criteria for linearity over the portion of the curve where tests were performed.

From these curves the relative sensitivity of the three procedures was compared. To obtain a minimal response of 1 mg. of glycogen per 10 gm. of mouse with method A 50μg. of compound E are required, for the same response with method B 2.1μg. are required, and with method C 0.8μg. are necessary.

V. *Liver sugar values with Compound E.*

In the interests of greater accuracy the standard curve for the four-day sugar method was set up in randomized block design as follows. After adrenalectomy five mice were placed in each cage and injected with oil or one of the four dilutions of Compound E, namely 5, 10, 20 and 40μg. There were sixteen mice on each dose, or eighty mice in all. Table 4 shows the mean sugar response at the various doses used, with their standard errors.

VI. *Urinary extracts.*

When urinary extracts were assayed by method B, they yielded curves parallel to that given by Compound E by the same method. (Curve E of Fig. 1).

DISCUSSION

I. *Synthesis of glycogen versus maintenance of glycogen level.*

The Reinecke-Kendall test (A) is based on the observation that certain adrenal cortical steroids will cause the deposition of glycogen in the liver of a glycogen-depleted adrenalectomized rat. It was observed in the experiments herein reported that after the sixteen hour fast used to deplete the reserve of liver glycogen, the animals, both rats and mice, were often in poor condition. Some appeared clinically to be in a state of hypoglycemia; prompt relief of symptoms followed the injection of glucose. The sixteen hour preliminary fast was therefore omitted in the hope of securing a measurable glycogen response from extracts of low potency. Mice were substituted for rats, as it was felt that the same dose of active material might produce a greater response in the smaller animal.

Instead therefore, of attempting to measure the rate of glycogen production, a test was devised to determine the potency of adrenal cortical steroids or urinary extracts in terms of their ability to maintain liver glycogen at a measurable level. Animals not subjected to the preliminary sixteen hour fast were found to have high levels of liver glycogen (Fig. 3), which varied over a wide range. This was thought to be due to differences between the time each animal had last eaten and the time when it started on the fast. The priming injection of adrenal cortical extract in method C was introduced in an attempt to build up a maximum level of liver glycogen. The initial level was raised from 14 to 26 mg. per 10 gm. of mouse, with a much narrower range of individual variation, Fig. 3. This was an advantage since it gave a much higher and more uniform starting level, which required less material for maintaining a measurable amount of glycogen.

II. *Total sugar versus glycogen determination.*

It was felt that the use of the fermentable sugar determination in

place of the glycogen analysis was justified, since a linear response with Compound E is obtained with doses up to 20μg. When semi-logarithmic coordinates were used, the sugar method (D) resulted in a straight line over a wider range of dosage than did the glycogen method (B).

III. *Sensitivity of the various methods.*

The sensitivity of method A on mice was compared with that of method B. Adrenal cortical extract (Upjohn) was used. In a series of twenty-one mice tested by method A, 0.2 ml. of the extract were required to produce an average liver glycogen of over 1 mg. per 10 gm. of mouse. By method B, the minimum dose of extract required to maintain this level was found to be 0.02 ml. in a series of eighteen mice. The new method, therefore, is about ten times as sensitive as method A with mice. A small series of rats was tested and found to be only about one-sixteenth as sensitive as mice to method A.

Attempts to use adrenal cortical extracts as a standard met with failure, as these extracts deteriorated rapidly after exposure to air. Compound E was therefore chosen as a standard since it is a stable pure compound.

Compound E was found to be more effective in the inhibition of glycogenolysis than in glycogenesis. With the glycogenesis method, A, 50μg. of Compound E were necessary to produce a liver glycogen of 1 mg. per 10 gm. of mouse. The same glycogen level was maintained with 2μg. of Compound E in method B, and with only 0.8μg. of Compound E in method C. Because of the large variations inherent in method C, that procedure has been used only for determining the presence and approximate degree of activity of small amounts of pure compounds, such as 1 to 10 mg., when enough material for titration is not available.

IV. *Comparison of precision of the glycogen method (C) and the sugar method (D).*[4,5]

The square of the average standard deviation in method D without the randomized block design is 8.4 as compared to 13.7 in the glycogen method C, a statistically significant difference. The addition of the block design to the total sugar method decreased the figure 8.4 to 6.9. The conclusion is that the variability in response in method D without the block design is 60 per cent of the variability in response in method C. The total sugar method plus the block design gives approximately half as much variation in response as method C without the block design.

The superiority of method D over method C is also shown by the

[4] Statistical analysis by Ruth F. Salt, Biometrician, Statistics Department, Memorial Hospital. See appendix for methods.

[5] Bliss, 1945; Fisher, 1942, 1943, 1944; and Snedecor, 1940.

greater slope, 21.0 as compared to 8.1 obtained in method C, a significant difference. (Fig. 5).

A comparison of the accuracy of potency determinations using varying numbers of animals on Standard and Unknown is given in Table 2. It is clear that potency can be estimated as accurately with

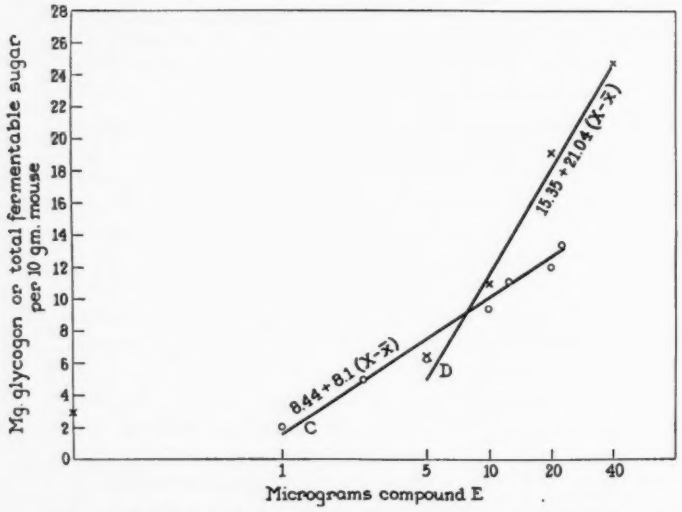

FIG. 5. The standard dose-response curves for methods C and D.

TABLE 5. EXACT CONFIDENCE LIMITS FOR MOST PROBABLE DOSE PREDICTED FROM RESPONSE USING METHOD D

	Most Probable Dose	Exact limits at .05 point	
		Lower	Upper
Log Dose	.72511	.44929	.85460
Dose	5.31	2.86	7.43
Log Dose	.94377	.79269	1.02373
Dose	8.77	6.17	10.52
Log Dose	1.33350	1.25823	1.47172
Dose	21.60	18.14	29.80
Log Dose	1.59966	1.46398	1.88985
Dose	39.80	29.10	78.01

5 animals per dose by the sugar method D as with 50 animals per dose by the glycogen method C.

When dose is estimated from response on the sugar curve, (Fig. 2 and Table 5), an estimated dose of 5μg. barely differs significantly from an estimated dose of 10μg., but a dose of 10 μg. easily differs significantly from a dose of 20μg. Above 20μg. the method again be-

comes less precise, probably because the liver is approaching saturation with carbohydrate.

V. *Urinary fractions—method B.*

When results for urinary extracts and Compound E were plotted the curves were found to be parallel. (See Fig. 1.) This suggests that the substances measured in the urinary extracts had activity very similar to that of Compound E. The results have been uniformly reproducible. When the same dilution was tested at intervals of several weeks the results have checked within 1 to 2 mg. per 10 gm. of mouse.

VI. *Evaluation.*

Method C has the advantage of great sensitivity when only very small amounts of material are available. However, the variations in results are so wide that large numbers of animals (twenty-five) are required for exact determinations of potency. Method B is sufficiently sensitive to test the activity of urinary extracts representing a twenty-four hour collection period. This method has been used to test activity of urinary extracts of normal persons and patients with various diseases. The method is simple to use and gives easily reproducible results.

Method D has not as yet been tested with urinary fractions; however, its greatly increased precision should render it the method of choice for testing pure compounds when only a few milligrams are available as well as for urinary extracts.

SUMMARY

Two highly sensitive methods for determining the glycogenic activity of steroids have been developed. In the titration method, (B) the amount of steroid necessary to maintain a liver glycogen of from 0.7 to 1.3 mg. per 10 gm. of mouse, is measured. In the priming method, (C), the activity of a steroid is compared directly with that of a known amount of Compound E. These tests depend upon the maintenance of liver glycogen in the fasting adrenalectomized mouse. They have been shown to be from twenty-five to sixty-two times as sensitive as those previously available and are adapted for the measurement of small amounts of steroid activity. Standard dose-response curves using Compound E as the reference standard were set up for both methods. The curves of suitably prepared urinary extracts were found to be approximately parallel to that of Compound E when tested by the same method. Method B has been shown to be applicable to the quantitative determination of small amounts of active substances in urinary extracts of unknown potency. Method C has been adapted to the detection of activity of small amounts of pure steroids.

A modification of the titration method (B) has been presented in

which the fermentable sugars are measured instead of the liver glycogen. The sugar method (D), has been analyzed statistically and found to have a precision of about ten times that of method C.

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APPENDIX

STATISTICAL ANALYSIS OF THE SUGAR METHOD, (D)

RUTH F. SALT[6]

Analysis of variance and exact confidence limits for most probable dose estimated from response. (Bliss 1945; Fisher 1942, 1944; Fisher & Yates, 1943; and Snedecor, 1940.)

The experimental design of 4 doses randomized in each of 16 blocks of mice, the mice in each block being given the same cage care permits the segregation of the sources of variation in response as given in the analysis of variance below.

Since the responses for 2 animals were missing, the total degrees of freedom were reduced from 63 to 61. One animal died; the second animal gave a response so high that the presence of remaining adrenal

[6] Biometrician, Statistics Department, Memorial Hospital.

TABLE 6. ANALYSIS OF VARIANCE FOR METHOD D

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Between blocks	15	188.51	12.57
Between doses	3	3,240.74	1080.25
Regression	(1)	(1,248.72)	(192.33)
Deviations from regression	(2)	(1,992.02)	(887.82)
Within doses (error)	43	298.51	$6.94 = s^2$
TOTAL	61	3,727.76	

tissue was suspected. (A response of 44.5 would occur by chance less than one time in 10,000 with the mean and standard deviation of 24.8 and 4.0 obtained for this dose level, omitting this suspiciously high response.)

While the mean square for blocks is not significant (Table 6), a worthwhile amount of variation was segregated from the variation within doses (error). If the block design had not been used, s^2 would be 8.4 instead of 6.9.

The formula for the curve fitted by the method of least squares is $15.35+21.04\ (X-\bar{x})$. Inspection of the mean squares in the analysis of variance above indicates that linearity is not in doubt and that regression differs significantly from zero.

The exact confidence limits at the .05 point for the most probable dose estimated from response are computed from the formula reported by Bliss (1945).

$$X_L = \bar{x} + C^2(Y-\bar{y})2/b \pm tC(s/b)\sqrt{(1/N+(Y-\bar{y})2/(B^2-s^2t^2)}$$

in which $B^2 = (Sxy)^2 : Sx^2$ and $C^2 = B^2 : (B^2 - s^2t^2)$. The most probable estimated dose and the approximate confidence limits are computed from the above formula omitting C^2, C, and s^2t^2. It is the exact confidence limits which are shown by the broken lines in Fig. 2 and in Table 5. In computing the significance of the difference between two estimated doses, the log dose and the confidence limits in log dose should be used.

NOTES AND COMMENTS

THE EFFECT OF PARTIAL HEPATECTOMY ON THE INACTIVATION OF α-ESTRADIOL

PINCUS AND MARTIN (1940) reported that the percentage of spayed female rats in which estrus vaginal smears were induced by 0.5 μg. of estrone was greater after hepatic damage produced by carbon tetrachloride than prior to it. Selye (1942), who employed the anesthetic effect of estrogens as an index, found that their activity is greatly increased by partial hepatectomy. We (1943, 1944) believe that our studies, which showed that more estrogen is required when injections were made intrasplenically than subcutaneously, offer further proof that estrogens are inactivated in the liver. In order to test our interpretation further we extended our studies to rats in which partial hepatectomy was performed immediately prior to the injection of estrogen.

MATERIAL AND METHODS

Mature female rats, purchased from Maguran Farms, Birmingham, Michigan, were spayed three to four weeks prior to the study. They were

TABLE I. RESULTS OF THE INJECTION OF α-ESTRADIOL BY VARIOUS ROUTES

	Subcutaneous injection		Intrasplenic injection		
	Dose μg.	Estrus %	Dose μg.	Estrus %	Ratio SC:IS
Controls	1.5	30	50	0	
	1.75	50	75	50	1:43
	3.0	85	150	90	
Partial Hepatectomy	1.0	25	10	30	
	1.25	50	12.5	50	1:10
	1.75	90	15	80	

primed with 50μg. of estrone in 0.1 cc. of peanut oil injected subcutaneously. Partial hepatectomy was performed by the method of Waelsch & Selye (1931) fourteen days after the priming injection. Immediately after removal of the liver the test injection of α-estradiol was given in 0.05 cc. of 5 per cent benzyl alcohol in sesame oil. Vaginal smears were taken with a moistened, cotton tipped toothpick, 48, 54, 60, and 72 hours after the injection. The smears were air dried and stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times

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was classified as representing a positive vaginal estrus response for that animal. Twenty animals were employed at each of the levels reported in Table I.

RESULTS

The results are presented in Table I. When α-estradiol is administered subcutaneously, the amount which is required for the production of estrus in 50 per cent of the test animals is reduced by approximately one-third when partially hepatectomized animals are used. The effect of partial hepatectomy is even more striking when α-estradiol is injected intrasplenically. In this instance the amount required for 50 per cent estrus is only one sixth that required in intact animals. The ratios of the amounts required for a 50 per cent estrus response are 1:43 and 1:10 (subcutaneous to intrasplenic) for the normal and partially hepatectomized animals respectively.

DISCUSSION

As anticipated from the work of Selye (1942) and Pincus and Martin (1940) the effectiveness of subcutaneously administered α-estradiol as measured by the vaginal response is increased by removal of most of the liver. The amount of α-estradiol required by intrasplenic injection for the production of estrus in 50 per cent of the animals is very greatly reduced by partial hepatectomy. Since the reduction in the amount of intrasplenically injected estrogen is greater in magnitude than the reduction in subcutaneously injected estrogen, the ratio of subcutaneous to intrasplenic is greatly reduced. This indicates a reduction in the amount inactivated by the liver.

SUMMARY

Partial hepatectomy in the spayed female rat reduces the amount of α-estradiol required to produce vaginal estrus in 50 per cent of the animals when the estrogen is injected either subcutaneously or intrasplenically. The reduction is greater when the estrogen is administered by the intrasplenic route. These facts are interpreted as additional evidence that entrogens are inactivated in the liver.

ACKNOWLEDGEMENTS

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ADMINISTRATION OF PROGESTERONE IN THE FORM OF MICROCRYSTALS

THERE is a trend in modern hormonal therapy to seek for slow absorption and therefore prolonged hormonal activity. Such is the rationale for the use of protamine-insulin, adrenalin-in-oil, esterified sex hormones and more recently the implantation of pellets of the sterol hormones. However, the necessity of a surgical procedure constitutes an inconvenience to the pellet method. It would therefore be a technical advance to obtain sterol hormones in a form which could be injected and which would be slowly resorbed. An aqueous emulsion of microcrystals would seem to have these properties. We have shown, in a series of experiments, that this occurs with microcrystals of progesterone.

METHODS

Microcyrstals of progesterone were obtained by dissolving pure crystalline progesterone with melting point of about 128° C. in pure dioxane. This solution was poured into distilled water, which contained about 0.5 per cent triethanolamine. The resulting emulsion was then allowed to remain until the crystals formed were well settled. The supernatant clear liquid was removed by suction in order to obtain the required concentration of the emulsion. In our experiments we used a concentration of 4.5 mgs. of progesterone in 1 cc. After shaking, the emulsion was fine enough to pass easily through an injection needle with a gauge of 20 to 21.

In our experiments we tested the emulsion on infantile female rabbits, pretreated for eight days with 10 I.U. of estrone daily (Clauberg test).

The emulsion was applied by a single subcutaneous injection in amounts ranging from 0.5 to 10 mg. of progesterone. The results were compared with the activity of solutions of pure crystalline progesterone in oil, ranging from 1 to 10 mgs. applied in single and individed doses. Biopsies of the uteri were made five and ten days after the first, or single injection of progesterone and sectioned for histological study.

RESULTS

The rabbits injected with 1 mg. of progesterone in oil, divided into five doses, showed a complete progestational transformation of the uterine mucosa—the day after the last injection. Five days later the uterus had completely regressed in the same animals.

After a single injection of 1 mg. of progesterone in oily solution the reaction of the uterine mucosa was entirely negative, showing only the influence of estrone without progestational proliferation. After 2 or 10 mgs. respectively

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were administered in a single injection, a clear progestational transformation of the endometrium was always obtained, but five days later (ten days after the injection) the progestational proliferation had regressed.

With microcrystals, we found that 0.5 mgs. produced a full development of the progestational uterine mucosa. One, 5 and 10 mgs. respectively were followed by the same effect. Five days later (10 days after the injection) the rabbits injected with 1 mg. of microcrystals showed a negative uterine test. whereas those which had received 5 and 10 mgs. had still a markedly positive progestational uterine mucosa.

Based upon these observations on 18 rabbits, we may say that 0.5 mgs. of progesterone applied in the form of microcrystals has at least as much effect on the rabbit uterus in the Clauberg test as does 1 mg. in oily solution given in 5 fractionated doses,—and as much activity as two milligrams in oil administered in a single injection. Thus, to obtain a minimum effect in the Clauberg test by one single injection, progesterone in microcrystals is at least four times as active as in oily solution.

Furthermore, we have shown that, probably due to slower absorption, there is a marked prolongation of the progesterone effect. The uterus of rabbits injected with 5 and 10 mgs. of microcrystals showed a marked progestational reaction ten days after the administration. Such prolongation obviously occurs in proportion to the dose, since rabbits injected with 1 mg. of microcrystals showed no response at 10 days, rabbits with five mgs. had a still positive although regressing mucosa, whereas rabbits treated with 10 mgs. had after ten days a fully developed endometrium.

Our observations indicate that this form of treatment with progesterone could be of value in instances where a prolonged progestational effect is required, as for example in habitual abortion.

SUMMARY

Progesterone in microcrystals in aqueous suspension, due to its slower resorption, is more efficient and more persistent in its activity in the Clauberg test, than is progesterone in oil solution.

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A STUDY OF PROGESTERONE INACTIVATION BY THE LIVER AFTER INTRAPORTAL ADMINISTRATION

THERE is some contradiction in the studies concerning the inactivation of progesterone by the liver. Progesterone is inactivated in the living body. Liver in vitro does not inactivate progesterone, as has been shown by Zondek and by our own experiments. The results of "in vivo" application of progesterone are somewhat contradictory. Mussio-Fournier, Morató-Manaro and Albrieux found progesterone injected into the liver or spleen of rabbits less effective in inducing progestational changes than when injected subcutaneously, Fels and Monacó did not confirm these experiments. Kochakian, Haskins and Bruce found progesterone pellets implanted into muscles or

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under the skin much more active than if they were implanted into the mesentery. Selye and Selye and Stone observed that the anesthetic action of orally administered progesterone is greatly enhanced by partial hepatectomy. Recently Masson and Hoffman observed that progesterone is by oral application in partially hepatectomized animals much more active than in normal controls.

In collaboration with Koref we described recently a method for the application of progesterone in form of microcrystals in aqueous emulsion. This aqueous emulsion can be applied by intravenous application (in animals). We used this form of progesterone injecting it into the portal vein, so that it had to pass through the liver, probably it was detained in the liver tissue and slowly resorbed.

The method was very simple. We used infantile female rabbits, treated for eight days with 10 I.U. of estrone daily (80 I.U.) in order to prepare them for the Clauberg test. The day after the last injection of estrone, the portal vein was laid open by laparotomy and the progesterone microcrystals injected directly into it. The abdominal cavity was then closed. We lost one animal by a hemorrhage from the portal vein after the injection.

Doses of 0, 5, 1, 2, 3 and 5 mgs. respectively were injected by this method into ten rabbits. Nine controls were treated by subcutaneous injection of progesterone in microcrystal emulsion, and nine other by equal doses of progesterone in solution in oil. The animals treated with 2, 3 or 5 mgs. all showed progestational transformation of the endometrium, and the histologic picture was not different from animals treated with equal doses of progesterone by different routes of administration. Of three animals treated with 1 mg., two showed a clearly progestational endometrium, whereas the uterine mucosa of the third was not transformed, but showed only the influence of estrone.

The animals treated with 0.5 mgs. were negative. Rabbits treated with 0.5 or 1 mg. respectively of progesterone in one single injection of oily solution were also negative, but rabbits treated with 0.5 or 1 mg. of progesterone in microcrystals by subcutaneous injection developed positive reactions. Therefore we can conclude that, although progesterone in microcrystals is as active by intraportal administration as in oily solution, by subcutaneous injection the same microcrystal emulsion is considerably more active. Doses higher than one mg. are not inactivated by intraportal injection, and one mg. of progesterone was in some animals active and in others not. One half mgs. were inactive by portal injection, but active when injected subcutaneously.

Some inactivating power of the liver on progesterone seems therefore to exist, but nevertheless much less inactivation by the liver occurs than with other sex hormones in vivo.

SUMMARY

Progesterone in microcrystals was injected to infantile rabbits in the Clauberg test by the subcutaneous route and by injection into the portal vein. The progestational activity of progesterone applied intraportally was inferior in low amounts (0.5 or 1 mg.). Doses above 2 mgs. were fully active. Some inactivation of progesterone by the liver in vivo seems to occur.

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